

## ANNUAL REVIEW OF BIOCHEMISTRY



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# ANNUAL REVIEW OF BIOCHEMISTRY

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## PREFACE

These paragraphs are being penned in early May, 1945. The European war has ended. The time for reconstruction has arrived. The bonds of fraternity and understanding which, in an earlier year, united scholars everywhere have yet to be restored. So also the sense of intellectual freedom without which the dispassionate and unrestricted pursuit of scientific inquiry is almost impossible. The restoration of universities, of scientific institutes, and of their libraries should be considered an essential part of the early efforts to reconstruct a shattered world. Despite all that has yet to be done in preparation, the day of complete international collaboration in the affairs of man is again at hand.

We of the *Annual Review of Biochemistry* have a clearly defined obligation that must speedily be met. Distribution of the *Review* in those countries that have been either devastated or isolated by war must be effected soon and forthcoming volumes must be so planned as to permit an increasing measure of collaboration in authorship on the part of our colleagues abroad. As for the former, several hundred copies of the *Annual Reviews* have been given to the American Library Association for institutional distribution abroad. As for the latter, invitations to biochemists in Europe and elsewhere will be extended as rapidly as facilities for collaboration are re-created. Volume XV will contain reviews prepared by several of our colleagues in Switzerland and Sweden. To assist them in their task we venture to request that reprints of papers published in the biennium ending June 30, 1945, and pertaining to the following fields, in particular, be sent to this office for forwarding to Europe: nonproteolytic, nonoxidative enzymes; the heme pigments; nucleic acid, nucleotides, purines, and pyrimidines; steroids and steroid hormones; triterpenoids. Reprints should be received here by August 31, if possible.

The present volume is the largest we have yet published. This does not necessarily reflect any substantial increase in the number of papers awaiting review. It is due rather to the fact that there were no withdrawals from authorship, and that several of the reviews which were unexpectedly long did not permit of appreciable curtailment in the editorial office.

Again we are privileged on behalf of those who use the *Review* to express a very sincere note of thanks to all who collaborated in au-

thorship. The task of the author in 1944 was no less difficult than in the earlier years of the war: the same unremitting pressure upon his time, and the continued difficulty of gaining access to much of the literature deserving of review. We would also express our thanks to those who assisted with helpful suggestions in respect to content, to the members of our office staff for their faithful and skillful assistance, to Professor Hubert Loring and Dr. Paul Boyer for much editorial aid, and to our printers, the Stanford University Press, for their fine collaboration throughout.

In answer to occasional inquiries it is perhaps proper to mention from time to time that Annual Reviews, Inc., is a non-profit corporation, established to publish *Reviews* such as the *Annual Review of Biochemistry* and the *Annual Review of Physiology*. Salaries are paid to the office and editorial staff, but members of the Board serve without compensation. The earnings that are made in favorable years are used to create a reserve against the less fortunate operations of the lean years, to maintain the selling price of the *Reviews*, irrespective of size, at \$5.00, and to further in every way the purposes for which the *Reviews* were established.

H. J. A.

C. L. A. S.

D. R. H.

J. H. C. S.

J. M. L.

H. A. S.

## ERRATA

Volume XIII, page 28, line 21: *for (heterophosphatase), read (heterophosphatase).*

Volume XIII, page 207, line 9 from bottom: *for Fischer, H. O. L., read Fischer, F. G.*



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# BIOLOGICAL OXIDATIONS AND REDUCTIONS

By H. A. LARDY<sup>1</sup> AND C. A. ELVEHJEM

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The journals available from abroad have continued to be limited in number during the past year and there has been a decrease in the amount of material reported on the general subject of "Biological Oxidations and Reductions." This condition has permitted the inclusion of a somewhat wider variety of material in the present review than has usually been covered. It has been gratifying to find an increased number of papers dealing with plant enzymes and respiration and this material will be included. The oxidation-reduction aspects of nitrogen fixation, the oxidative enzymes of tumor tissue, and the oxidation of the naturally occurring amino acids will be discussed in other chapters.

## IRON PORPHYRIN-CONTAINING ENZYMES AND CARRIERS

*Cytochrome and cytochrome oxidase.*—Outstanding among the past year's achievements in the field of plant respiratory enzymes has been the isolation of cytochrome-*c* and the further purification of cytochrome oxidase from wheat germ by Goddard (1). Cytochrome-*c* was obtained in solution by extracting solvent-defatted wheat germ powder with 0.1*M* dipotassium hydrogen phosphate. Proteins of larger particle size were removed by acid precipitation and subsequent treatment of the neutralized solution with solid ammonium sulfate. Cytochrome-*c* was precipitated at 0° C. and pH 3.8 by addition of trichloroacetic acid. The sediment was washed with concentrated ammonium sulfate solution, its solution was dialyzed in the cold, and the protein was precipitated with acetone. The precipitate was washed with acetone and dried in a vacuum desiccator. The absorption spectrum of the cytochrome-*c* which was extracted from this powder with cold water could not be distinguished from that of cytochrome-*c* from beef heart. The wheat germ cytochrome-*c* was catalytically oxidized by cytochrome oxidase from either heart or wheat and was reduced by the cytochrome reductase system of Haas *et al.* (2). In low concentrations, wheat germ cytochrome-*c* was as active as heart cytochrome-*c* in catalyzing the oxidation of hydroquinone in the system hydroquinone-cytochrome-*c*.

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cytochrome oxidase, but at high concentrations the maximum rate of oxidation attained with wheat cytochrome-*c* was about 30 per cent less than with heart cytochrome-*c*. The presence of an inhibitory substance in the preparation from wheat germ could not be demonstrated. The amount of cytochrome-*c* in wheat germ was calculated to be sufficient to account for the respiration observed. Goddard postulates that the free heme in wheat germ (demonstrated by the appearance of a hemochromogen spectrum when intact germ or extracts are treated with hydrosulfite and pyridine) may be used for the synthesis of cytochrome-*c* during germination when the respiratory activity increases greatly. It will be interesting to see whether cytochrome-*c* from plant sources resembles animal cytochrome-*c* in such properties as amino acid composition, magnetic susceptibility, etc.

Cytochrome oxidase activity was demonstrated in the alkaline phosphate extract of wheat germ powder by Goddard (1), and could be separated entirely free from cytochrome-*c* by precipitation with acetate buffer. The oxidase remained in the supernatant solution when the alkaline phosphate-wheat germ powder suspension was centrifuged for thirty minutes at 3000 *g* but was largely sedimentated by 10,000 *g* for 100 minutes. The oxidase could not be separated from this sedimentable material after the ultrasonic treatment which was found by Haas (3) to allow solution of heart cytochrome oxidase. Succinic dehydrogenase was also present in the crude cytochrome oxidase preparations but could be preferentially inactivated by ageing or precipitation with acetate buffer.

Cytochrome oxidase inhibitors decreased the respiration of the flavedo tissue of the orange fruit, while the addition of cytochrome-*c* stimulated the respiration (4). The absorption band characteristic of cytochrome-*b* was detected spectroscopically in the tissue after treatment with hydrosulfite.

A notable advance in the study of the "respiratory enzyme" has been made by Haas (5) who has now succeeded in separating the cytochrome oxidase of pig heart (3) into a heat-labile, sedimentable fraction (Component I) and a heat-stable, soluble fraction (Component II). Component I was sedimentated by centrifugation for two hours at 10,000 RPM and contained only a small quantity of Component II. The latter was prepared by placing an undiluted preparation of cytochrome oxidase in boiling water three minutes, by centrifuging off the denatured protein and by washing the residue with ammonium buffer at pH 9. The supernatant solution and wash liquid

were combined and centrifuged for two hours at 10,000 *g*. The water clear supernatant contained 72 per cent of the original Component II activity. Component II is entirely inactive in the oxidation of reduced cytochrome-*c*; Component I has some activity, probably because it contains a small amount of Component II, and the two fractions combined show activity approaching that of the original oxidase preparation.

Using the Nadi reagent as a means of detecting cytochrome oxidase, Moog (6) reported this enzyme to be present in chick embryo as early as the head process stage. The content of this "indophenol oxidase" increased in quantity to the second day of incubation and remained at this level until the fourth day. The increased quantity of cytochrome oxidase in the chick embryo on the fourth day of incubation permits its detection by manometric methods (7).

Schachner, Franklin & Chaikoff (8) found that inhibitors of cytochrome oxidase prevent the formation of diiodotyrosine and thyroxine from inorganic iodide by surviving thyroid slices. The formation of organic iodine compounds appeared to occur in two stages. The first, an accumulation of inorganic iodide by thyroid tissue, was inhibited by cyanide or sulfide but not by azide or sulfa drugs. The conversion of accumulated inorganic iodide to organic form was prevented by each of these inhibitors (9). A direct relationship has been reported between the activity of the rat or guinea pig thyroid *in vivo* and its "oxidase" activity, as measured with *p*-phenylenediamine (10). Adrenalectomy led to a decreased cytochrome oxidase activity in the heart, kidney, and liver tissue of rats (11). Decreases in cytochrome-*c* content of the liver and kidney were also observed in adrenalectomized rats (11).

Two of the six atoms of sulfur in each molecule of cytochrome-*c* were found by Åkesson (12) to occur as methionine. No inorganic sulfate is present and the other four sulfur atoms probably occur as cystine but this has not yet been definitely established. The chemistry and function of cytochrome were reviewed by Zeile (13). Lyophilization of cytochrome-*c* and subsequent storage for four months produced no changes in absorption spectrum or enzymatic activity (14).

*Catalase*.—Yamafuzi *et al.* reported (15) that sugar cane leaves infected with virus have a lower catalase activity than normal leaves, and that infected areas of a given leaf contained less catalase than a healthy portion of the same leaf. A somewhat similar situation oc-

curs in animal tissues. Greenstein and co-workers (16, 17) reported a reduction in catalase activity in the livers of mice and rats bearing tumors. No decrease in liver catalase activity occurred in the livers of mice bearing actively growing embryonic implants or during pregnancy (18). Hemoglobin levels may also be decreased during tumor development (19), and Greenstein (20) has suggested that the presence of a tumor interferes with the synthesis of hematoporphyrin used in formation of both catalase and hemoglobin. Yamafuzi *et al.* (15) believe that leaf catalase disappears because it is included in the virus protein molecule.

By determining the rate constants at various temperatures for the decomposition of hydrogen peroxide by crystallized catalase, Sizer (21) was able to determine the optimum temperature for the enzyme's activity ( $53^{\circ}$ ), the activation energy of the enzyme-catalyzed reaction, and the activation energy for the heat inactivation of catalase. In the range from  $2$  to  $40^{\circ}$  the initial decomposition of peroxide by catalase was described by the equation for a zero order reaction and it was calculated that the activation energy of the reaction was 4200 calories per gram molecule. Heat inactivation of catalase increased with temperature in accordance with the Arrhenius equation; from kinetic data the activation energy of the reaction was calculated as 55,200 calories. Heat inactivation of catalase was also studied by incubating the enzyme at various temperatures for five minutes in the absence of substrate and subsequently determining the activity at  $35^{\circ}$ . From the data obtained by this method the activation energy of the heat inactivation of catalase below  $62^{\circ}$  was found to be 55,000 calories (confirming the value obtained from kinetic data) while from  $62$  to  $68^{\circ}$  the value became 255,000 calories. The velocity constant for the decomposition of hydrogen peroxide by catalase was found to be  $3.4 \times 10^5$  liter mole $^{-1}$  sec. $^{-1}$ , which Sizer points out is similar to that of a number of other heme-protein catalyzed reactions.

A note on the polarographic behavior of catalase was published by Brdička *et al.* (22).

*Peroxidase.*—Heat inactivation of peroxidase was found by Schwimmer (23) to resolve the enzyme into a soluble fraction and a heat precipitated fraction which later recombine and revert to the active enzyme. The regeneration of the enzyme is a time reaction and was more nearly complete after twenty hours incubation at  $25^{\circ}$  than at  $6^{\circ}$ .

*Miscellaneous iron-porphyrin compounds including hemoglobin.*—

Gibson (24) found that, contrary to the report of Vestling (25), the methemoglobin produced by ferricyanide is identical with that produced by nitrite. Ferricyanide methemoglobin was found to be more rapidly reduced by ascorbic acid than was nitrite methemoglobin because of the catalytic effect of traces of ferricyanide present in the former preparation. The reduction of methemoglobin by ascorbic acid was described by the equation for a dimolecular reaction (24).

Horecker & Brackett (26) described a spectrophotometric method for the determination of methemoglobin and carboxyhemoglobin in blood.

Simon, Horwitt & Gerard (27) found that hemoglobin, which catalyzes the oxidation of linoleic acid and of brain phospholipids, depresses the marked acceleration of this oxidation by ferrous-*o*-phenanthroline. The authors suggest that the hemoglobin adsorbed on the substrate may have displaced the more active catalyst and some evidence for this was found. This possibility could also be tested in experiments with varying ratios of catalysts to substrate.

The claim of Bechtold & Pfeilsticker (28) that myoglobin is the precursor of cytochrome was reinvestigated by Gonella & Vannotti (29) who found that purified myoglobin, which had been extracted from blood-free muscle, consists of a mixture of two hemochromogens—true myoglobin, and a protein-free hemin which is adsorbed on the myoglobin. They form pyridine-hemochromogens at pH 7 or above, with absorption bands at 565 and 555 mμ, respectively, and the spectrum of a mixture of the two is similar to that of a mixture of cytochrome-*b* and *c* which Bechtold & Pfeilsticker (28) originally believed to be formed. These results also explain the recent observations of Pfeilsticker (30) who found a weak absorption band at 552 mμ. in pyridine-treated oxymyoglobin and, recognizing that true cytochrome was not formed, named the substance myoglobin cytochrome-*c*<sub>52</sub>.

The synthesis of monoazohemochromogens by treatment of verdo-hemochromogens with ammonia at room temperature in the absence of oxygen was described by Lemberg (31). Iron could be removed from the monoazo-heme compounds with hydrazine hydrate and glacial acetic acid yielding monoazoporphyrins (in which one of the methine groups linking the pyrrolic rings is replaced by nitrogen) which are identical with the "monoimidoporphyrins" synthesized by Hans Fischer. This investigation has not only confirmed the structure assigned by Fischer to these compounds, but has also presented a new approach to their study.

Klüver (32) discussed the distribution of porphyrins in the central nervous system of various species.

When grown on iron-deficient media, *Aerobacter indologenes* was found by Waring & Werkman (33) to be low in catalase and peroxidase, and devoid of hydrogenase, formic dehydrogenase, and hydrogenlyase activity. These investigators suggested that hydrogenlyase (33a), which decomposes formic acid to molecular hydrogen and carbon dioxide, is not an individual enzyme but a three-component system of formic dehydrogenase-carrier-hydrogenase. They postulate the carrier to be an iron-containing protein whose absence from the iron deficient bacteria would result in their failure to exhibit these particular activities. Previous evidence that hydrogenase activity involves an iron compound was reviewed earlier by Lipmann (34) and further studies on its activation and inactivation were discussed by Gaffron (35).

#### OTHER HEAVY METAL-PROTEIN ENZYMES

*Polyphenolases.*—Nelson and co-workers have continued their studies on the copper-protein tyrosinase (36, 37, 38, 46) and Nelson & Dawson (39) have reviewed the pertinent literature. They investigated the physical properties of the enzyme and concluded that tyrosinase was a copper protein entity or complex possessing two enzymatic activities, the ratio of these activities depending on the size, shape, surface characteristics, etc., of the protein complex (39). Evidence was presented that tyrosinase becomes inactivated by the process of enzymatic activity itself rather than by any known products of the reaction. Several lines of evidence were presented by Behm & Nelson (37) that tyrosinase can exhibit monophenolase activity (catalysis of phenol oxidation in this case) only when it is simultaneously catalyzing the oxidation of an *o*-dihydric phenol. In measuring phenolase activity of tyrosinase, it was found (36) that the rate of oxygen uptake, with phenol as substrate, agrees with the rate of phenol disappearance, this fact indicating that the subsequent oxidations of the *o*-dihydric phenol produced proceed at rates at least as great as the oxidation of phenol. Manometric methods are therefore suitable for measuring the monophenolase activity of tyrosinase with phenol as substrate. An improved method for the determination of tyrosinase catecholase activity has been developed which is based on the initial reaction velocity (46).

The polyphenolase of Ceylon tea (40, 41, 42) has been found to be an iron-free copper-protein (42). The best preparation obtained,

evidently bearing considerable inert material, contained 0.08 per cent copper and 6.6 per cent nitrogen; its activity, per unit weight of copper, was about one-tenth that shown by Kubowitz' potato oxidase (43). Oxidase activity paralleled the copper content of the more purified enzyme preparations and removal of the copper by dialysis against potassium cyanide irreversibly inactivated the oxidase. The tea oxidase is not an ascorbic acid oxidase (40) and exhibits no cytochrome oxidase activity (41).

A comparison has been made of the oxidase activity, toward a number of substrates, of normal and tumor beet root tissue (44).

Free *L*-tyrosine has again been isolated from potato tubers (45, 38), and its role in the respiration and darkening of potatoes discussed. The effect of various heavy metals (47) and of heat (48) on the pro-tyrosinase of grasshopper eggs was reported.

*Ascorbic acid oxidase*.—Powers, Lewis & Dawson (49) reported the preparation of a highly purified ascorbic acid oxidase from the press juice of the summer squash (*Cucurbita pepocondensa*). The oxidase activity was proportional to the copper content of the preparations, and the final preparation contained 0.24 per cent of the metal. This preparation is one and one-half times as active as one previously reported (50) to contain 0.15 per cent copper. In dilute solution the highly purified preparation lost activity rapidly unless an inert protein was present. The pH optimum for the diluted enzyme in citrate-phosphate buffer, either with or without added gelatin as stabilizer, was about 5.6.

The inactivation of the enzyme during manometric experiments was found to be the result of environmental factors (preventable by inert protein) and some factor inherent in the ascorbic acid-ascorbic acid oxidase-oxygen system (51). The latter factor could not be attributed to hydrogen peroxide or to dehydroascorbic acid but was eliminated by the addition of small amounts of catalase, peroxidase, methemoglobin, or hemin to the manometer flasks. Inhibition of catalase or peroxidase activity also prevented the protective action of these respective substances on the ascorbic acid oxidase.

Diemair & Zerban (52) purified the ascorbic acid oxidase of cucumber and pumpkin and also found the copper content to parallel the activity. Loss of copper during prolonged dialysis paralleled the irreversible loss of enzyme activity.

Crook & Morgan (53) observed the occurrence in the juice of several plants of their previously reported enzyme which catalyzes the



reduction of dehydroascorbic acid by reduced glutathione. Bukin (54) also reported that ascorbic acid oxidase is not the enzyme which catalyzes this reaction. He proposed the name "ascorbic reductase" for the enzyme which does catalyze the reduction and suggested a function for it in the scheme of hydrogen transport.

Catalysis of the aerobic oxidation of aromatic hydrocarbons by ascorbic acid has been reported (55) and dehydroascorbic acid appears to be the oxidant.

*Iron-protein complexes.*—Libet & Elliott (56) described an iron-protein complex, ferrin, obtained from heated rat, rabbit, guinea pig, hog, or beef livers. In partially purified form, it contains 15.7 per cent of ferric iron, is soluble in neutral or alkaline solution, is precipitated below pH 6.7, shows no absorption bands in the visible range and does not form a hemochromogen. It probably is a denatured protein since it is obtainable only from heated liver, and is not derived from ferritin, since it can not be obtained from spleen. Ferrin catalyzes the oxidation of crude phospholipids (57).

#### DEHYDROGENASE AND OXIDASE SYSTEMS

*Flavin-containing systems.*—The *d*-amino acid oxidase of *Neurospora* (58) deaminates some nineteen *d*-amino acids with optimum activity at pH 8.0 to 8.5, and on amino acids with chain lengths of 5 carbon atoms. It differs strikingly from the enzyme prepared from animal tissues in that it is not inhibited by 0.01 *M* benzoate.

Neuberger & Sanger (59) have found that, although *d*-lysine is not attacked by *d*-amino acid oxidase,  $\epsilon$ -acetyl- or  $\epsilon$ -benzoyl-lysine is oxidized at a moderate rate.  $\epsilon$ -Methyllysine was not oxidized. They postulate that the free basic group in the  $\epsilon$  position may inhibit the enzyme by repelling another basic group on the oxidizing enzyme.

Atabrine inhibits *d*-amino acid oxidase and the oxidation of glucose, lactate, pyruvate, malate, or citrate by a number of rat tissues (60). Haas (61) used purified enzyme systems to determine the site of action of the drug and found it to inhibit cytochrome reductase, glucose-6-phosphate dehydrogenase, and, to a slight extent, cytochrome oxidase. Additions of one  $\mu$ g. of riboflavin phosphate counteracted the inhibition of cytochrome reductase by 500  $\mu$ g. of atabrine.

Flavoproteins are probably concerned in the luminescence of the luciferase system (62, 63). The synthesis of flavin adenine dinucleotide by tissues from normal and flavin-deficient rat tissues was studied (64).

During the oxidation of xanthine by Schardinger enzyme in milk, added inorganic iodine was fixed in organic form (65).

*Other coenzymes.*—Anfinsen, using his own micro adaptation (66) of the method of Jandorf *et al.* (67) for the determination of diphosphopyridine nucleotide (DPN), has studied the distribution of this coenzyme in the bovine retina (68). An extremely high concentration of DPN was found in the synaptic regions where Anfinsen considered it to be important in the oxidation of carbohydrate to produce the pyruvate required for the synthesis of acetylcholine.

The smallest structural unit which can serve as V-factor for the *Hemophilus* organism is nicotinamide riboside (69). It was slightly more active than triphosphopyridine nucleotide but considerably less active than DPN. Desamino DPN was about 60 per cent as active as DPN itself.

Hoagland and co-workers (70) find that the ingestion of nicotinic acid increases the V-factor content of human erythrocytes and also the ability of the erythrocyte to oxidize malate and lactate, while ingestion of nicotinamide produced no such increases. Anderson, Teply & Elvehjem (71) found that the DPN content of chick's voluntary muscle was correlated with their intake of dietary nicotinic acid.

Although antagonism between sulfa drugs and nicotinamide metabolism has been frequently demonstrated, Anderson, Pilgrim & Elvehjem (72) found sulfapyridine and sulfathiazole not to inhibit DPN-linked systems in yeast or animal tissue preparations. The inhibition of DPN-linked systems by salicylate was studied by Euler & Ahlström (73). Information on the nicotinic acid-containing coenzymes was reviewed (74). Gunsalus, Bellamy & Umbreit (74a) discovered that a phosphorylated form of pyridoxal is a coenzyme for the decarboxylation of tyrosine by a bacterial enzyme preparation. Pyridoxal was phosphorylated either enzymatically by adenosinetriphosphate (ATP) or by chemical means to produce the active coenzyme.

*Fatty acid oxidation.*—Confirmation of the occurrence in liver of a higher fatty acid dehydrogenase requiring muscle adenylic acid as a coenzyme (75) has again been offered (76). Although the necessity of inorganic phosphorus in this reaction (77) indicates a possible coupling of the dehydrogenation with phosphorylation, this seems not to have been investigated.

Mazza & Marfori (78) reported that a phosphate or bicarbonate extract of liver tissue contained a dehydrogenase, especially active on fatty acids with 12 or more carbon atoms, which did not require the

addition of a coenzyme for activity. Spectroscopic evidence was obtained that the dehydrogenation of stearic or oleic acids resulted in the formation of double bonds in the  $\alpha$ - $\beta$ -position, and the investigators point out the possible relation of the dehydrogenation to  $\beta$  oxidation.

Leloir & Munoz (79) have continued their investigations of the oxidation of butyrate by liver preparations which require additions of cytochrome-*c*, adenylic acid, inorganic phosphate, and a di- or tricarboxylic acid before catalysis of the oxidation of butyrate to acetoacetate can occur. During this oxidation of butyrate to acetoacetate, other substances are also oxidized, accounting for about three-fourths of the oxygen consumed. They have now found that during the oxidation of citrate, succinate, and malate by these preparations, phosphopyruvate is produced by a reaction that does not involve enolase and that added phosphopyruvate accelerates the oxidation of butyrate if bicarbonate is present. A point of interest is the demonstration that the greater part of the butyrate oxidation occurs after the phosphopyruvate has completely disappeared. These observations are of significance not only for the oxidation of fat but also for the mechanism of phosphopyruvate formation which will be discussed below.

The situation regarding fatty acid oxidation by liver preparations has been greatly clarified by the finding of Lehninger (80, 81) that adenosinetri- or di-phosphate (ADP) can replace the combination adenylic acid plus fumarate or pyruvate in activating the oxidation of fatty acids with from four to ten carbon atoms. In the absence of ATP the fatty acids inhibited the endogenous respiration of the liver preparations, which property Lehninger assigns to their surface activity. Acyl phosphates did not inhibit endogenous respiration and did not require additions of ATP in order to be oxidized. The possibility of the high energy phosphate group of the acyl phosphates being transferred to the adenylic system to act elsewhere was eliminated in other experiments (81) and the postulation made that dehydrogenation of fatty acids can occur in these liver preparations only if the carboxyl group is present as an anhydride with phosphoric acid.

Acetate was found by Kleinzeller (82) to be rapidly oxidized by guinea pig kidney cortex with the production of bicarbonate. The oxidation of acetate was completely inhibited by 0.031 *M* malonate, and since glycolic and glyoxylic acids were not appreciably oxidized, they were excluded as possible intermediates. The oxidation of acetate by bull spermatozoa furnishes energy for maintenance of motility (83).

The oxidation of butyrate and a number of closely related acids by

kidney tissue was also studied by Kleinzeller (84). Butyrate, crotonate, vinylacetate,  $\beta$ -hydroxybutyrate,  $\gamma$ -hydroxybutyrate, and *dl*- $\alpha\gamma$ -dihydroxybutyrate were rapidly oxidized with the production of bicarbonate in kidney slices from well-fed guinea pigs and rats. *trans*- $\gamma$ -Hydroxycrotonate was oxidized in rat but not in guinea pig kidney slices. The oxidation of each of these acids was inhibited by 0.031 *M* malonate. Since the rate of vinylacetate oxidation was equal to, or exceeded, that of butyrate, Kleinzeller concluded that it may be an intermediate in the complete oxidation of butyrate (84).

To continue the study of  $\alpha,\gamma$ -diketo acid metabolism, Lehninger (85) synthesized  $\alpha$ - $\gamma$ -diketo-*n*-octanoic acid and studied its metabolism both *in vivo* and *in vitro*. It was very poorly absorbed from the intestine of rats, not oxidized by rat liver slices, and oxidized by homogenized rat liver only very slowly.

Weinhouse, Medes & Floyd (86) proved in a striking experiment with heavy carbon that  $\beta$ -oxidation of octanoic acid can occur with subsequent condensation of the two-carbon fragments. Previous arguments, based mainly on the recovery of more than one mole of ketone-body per mole of fatty acid oxidized, against  $\beta$ -oxidation as the main pathway of fatty acid cleavage have been finally invalidated by these experiments which showed that the carboxyl carbon of octanoic acid is distributed equally between the carboxyl and carbonyl carbon atoms of the acetoacetate produced by surviving kidney slices. The ketone bodies did not arise, therefore, by "multiple alternate" oxidation of the fatty acid chain.

Studies on the enzyme lipoxidase have continued with the main interest centered on the nature of the enzymes as revealed by inhibitor studies. Hummel & Mattill (87), using crude extracts of soybeans as the enzyme source, found that cyanide, sulfide, carbon monoxide, and pyrophosphate inhibit oxygen consumption. Süllman (88), too, found the enzyme to be inhibited by cyanide and sulfide but did not find pyrophosphate to inhibit. Since cyanide did not inhibit the decolorization of 2,6-dichlorophenolindophenol by lipoxidase plus lard fatty acid esters in Thunberg experiments, Hummel & Mattill (87) concluded that the enzyme is either a dehydrogenase or is multiple in nature. Balls & Kies (89), however, found that an extract of soybean meal did not show dehydrogenase activity toward unsaturated fatty acid esters and argue against the dehydrogenase nature of the enzyme. Süllman (90) independently confirmed the findings of others [see (91)] that lipoxidase catalyzes the oxidation of the more unsatu-

rated fatty acids and their esters, and further found that the group  $\cdot\text{CH}:\text{CH}\cdot(\text{CH}_2)_7\cdot\text{CO}\cdot$  must be present in *cis* configuration. The enzyme occurs in legumes and potato press juice but not in the juices of some twenty other species of plants investigated. Discussion of the nature of this enzyme system best awaits further studies on purification of the active protein material.

Removal of the naturally occurring antioxidants from both plant and animal fats allows more striking demonstration of the antioxidant properties (tested at 37°) of added  $\alpha$ -tocopherol (92).  $\beta$ -Carotene shortened the induction period and accelerated the rate of oxygen consumption thereafter, especially of the more highly unsaturated fats. The antioxidant properties of N,N-dimethylaminoazobenzene were discussed by György & Tomarelli (93) in relation to the effect of various fats on its carcinogenicity.

Calkins & Mattill (94) conclude from kinetic studies that quinone synergizes the antioxidant properties of ascorbic acid in unsaturated fats by allowing the reduction of peroxides by ascorbic acid to proceed in a series of univalent oxidation-reduction steps, thus reducing the activation energy of the reaction. They propose that quinone is reduced by ascorbic acid to a semiquinone which is in turn reoxidized to quinone by the activated peroxide radical. The accumulation of the fatty acid peroxides is thus prevented.

Atherton & Hilditch (95), after isolating the decomposition products, concluded that gaseous oxygen acts on methyl oleate at 20° to form, largely, a hydroperoxide at the methylene group adjacent to the ethenoid bond, like that produced in the photochemical oxidation of methyl oleate at 35° (96). Hilditch (97) reviewed the recent work on antioxidants and discussed their practical applications.

*Other dehydrogenases and oxidases.*—To test further the concept that auxins exert their growth effects through action on respiratory enzymes (98), Berger & Avery (99) studied the effects of synthetic auxins on the glutamic dehydrogenase and *isocitric* dehydrogenase systems of the *Avena* coleoptile. The glutamic dehydrogenase required DPN for its activity while *isocitric* dehydrogenase depended upon triphosphopyridine nucleotide (TPN) and either  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$  or  $\text{Co}^{++}$  ions. None of the synthetic auxins accelerated the dehydrogenases and, at higher concentrations, some were inhibitory.

Breusch (100) found a series of dehydrogenases in cat liver which can oxidize the unphosphorylated forms of *d*-sorbitol, *d*-arabinose, probably *d*-erythrose, glyceraldehyde, glycolaldehyde, and also for-

maldehyde in Thunberg experiments with methylene blue as the oxidizing agent. Glucose, a number of sugar acids, and other sugar alcohols were not attacked. Oxidation of these sugars was not inhibited by calcium as were the dehydrogenases previously described (101) which transferred hydrogen from *d*-glucose, *d*-ribose, and glyceraldehyde to oxaloacetic acid. A glucose dehydrogenase from germinating seeds of *Phaseolus* was reported (102). It acts on glucose, mannose, and galactose but not on fructose and pentoses.

The inhibition of the succinoxidase system by sulfhydryl compounds (103) was studied in detail by Ames & Elvehjem (104) using the cysteine-cystine system. The inhibitory substance was shown to be cystine rather than some other oxidation product (105). The extent of inhibition obtained in tissue homogenates was shown to be dependent on the concentration of four-carbon dicarboxylic acids, specifically fumarate and malate. Malate and fumarate protected against the action of cystine (104), which fact is further support for the suggestion (103) that the active sulfhydryl groups of the enzyme are located on that portion of this enzyme's surface having affinity for dicarboxylic acids of suitable chain length.

Echinochrome A, the prosthetic group of a respiratory enzyme of the sea urchin's sperm, was synthesized by Wallenfels & Gauhe (106).

#### COUPLING OF OXIDO-REDUCTIONS WITH ENERGY UTILIZATION

As yet, the only known means of coupling oxidation-reduction reactions with energy utilizing processes is by the intercession of the system "high energy" phosphate<sup>2</sup>/inorganic phosphate. Whether, as in the case of muscle contraction, the adenosine polyphosphates are the ultimate phosphate donors in all energy utilizing systems remains to be disclosed. The search for means other than phosphorylation for coupling oxido-reductions with energy utilization should most certainly be encouraged, but the observation that, in *Fusaria*, sulfur acts as a hydrogen acceptor (108) does not in the opinion of the reviewers "corroborate the fact that there may be too much importance attached to the phospho-organic esters as overall intermediary transfer agents" (108).

*Efficiency of oxidative phosphorylations.*—Ochoa found that during the oxidation of pyruvate (109) or of  $\alpha$ -ketoglutarate (110) in heart extracts about three moles of inorganic phosphate were esterified

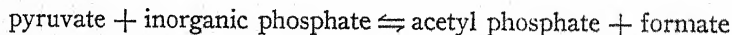
<sup>2</sup> For definition of this type of compound see Lipmann (107).

per atom of oxygen consumed. This figure was obtained after correcting the overall phosphorylation for the loss of esterified phosphate through phosphatase activity, by simultaneously determining the efficiency of the anaerobic oxidation of triose phosphate with pyruvate as the hydrogen acceptor. Thus in the complete combustion of pyruvate about 60 per cent of the free energy liberated is stored in the form of utilizable phosphate bond energy. While it has been known that the oxidative decarboxylation of pyruvate leads to the formation of acetyl phosphate (107), the mechanism of the formation of the other high energy phosphate bonds is as yet completely obscure. Ochoa found that the oxidation of chemically reduced DPN in these same extracts led to no phosphate uptake (109) and that the oxidation of succinate to fumarate led to the fixation of only one mole of phosphate per atom of oxygen consumed (111).

*The role of phosphate in various oxido-reductions.*—The oxidation of the lipid reserve (112) of bovine spermatozoa was found to lead to an uptake of inorganic phosphate with the production of a labile phosphate compound which appeared to be adenosinepolyphosphate (113). Inhibition of the respiration by cyanide caused a decrease in the acid-labile phosphate with an equivalent increase in inorganic phosphate, while addition of glucose to cyanide-treated spermatozoa allowed the anaerobic glycolytic system to esterify phosphate and maintain motility (113). This appears to be the first report of phosphate fixation during fat oxidation.

A new type of reaction involving the utilization of high energy phosphate was described by Binkley (114). An enzyme preparation from rat liver, requiring the addition of zinc ions, catalyzed the cleavage of cystathionine if ATP were added. The labile phosphate of the ATP was utilized in the reaction and did not appear in the usual inorganic fraction, but rather as an acid-stable ester, probably of homoserine. Cysteine was liberated in the reaction.

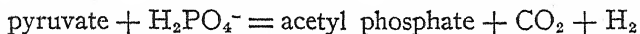
Much interest continued in the "phosphoroclastic" dissimilation of pyruvate by various bacterial enzyme preparations. The product of the reaction is acetyl phosphate rather than acetic acid (115, 116, 117). The reversibility of the reaction



was demonstrated by the distribution of isotopic carbon introduced as formate (118) and by the production of keto acid when both formate and acetyl phosphate were incubated with the extracts (119). Utter,

Werkman & Lipmann (118) found that  $C^{18}$  added in the formic acid was distributed in equal concentration between the carboxyl groups of pyruvate and formate. It was shown that the formate was not in equilibrium with carbon dioxide in the enzyme preparations used and therefore, that the labeled pyruvate had not arisen in their experiment by the addition of carbon dioxide to another compound. Their work demonstrates that, in addition to the well-known Wood & Werkman reaction, a second means of carbon dioxide fixation exists in heterotrophic organisms, for in the intact cell formate and carbon dioxide are in equilibrium.

Koepsell, Johnson & Meek (117) have continued their studies on pyruvate dissimilation by extracts of *Clostridium butylicum* which produce acetic acid, carbon dioxide, and molecular hydrogen. The reaction was again formulated as



because the forward reaction occurs only in the presence of an appreciable concentration of inorganic phosphate which is esterified and because of the production of silver precipitable acetic acid. A new metabolic product of great interest, butyryl phosphate, was produced in their preparations by transfer of phosphate from acetyl phosphate to butyric acid.

Potter (120) studied the oxidation of four-carbon dicarboxylic acids by highly diluted fresh homogenates of rat kidney tissue fortified with cytochrome-*c*, magnesium ions, sodium fluoride, chloride ions, ATP, and inorganic phosphate and with creatine as a phosphate acceptor. The oxidation of succinate, at all concentrations tested, led to a decrease in inorganic phosphate. Malate and oxaloacetate were likewise oxidized with a net uptake of inorganic phosphate, but when added in high concentrations they caused increases in the inorganic phosphate at the expense of the original ATP which was added to the flask. These results are probably related to the previously observed effect of oxaloacetate, or of pyruvate plus bicarbonate, in stimulating the dephosphorylation of ATP in aged muscle extracts (121).

Schneider & Potter (122, 123) studied the uptake of inorganic phosphate during the oxidation of pyruvate and  $\alpha$ -ketoglutarate by dilute kidney and brain homogenates. The keto-acid oxidase system was found by them to be extremely sensitive to pentobarbital (124).

Youngberg (125) found that kidney extracts fortified with adenylic acid, succinate, magnesium ions, and sodium fluoride will phosphory-



late glucose by an aerobic process but will not phosphorylate pentoses under the same conditions. The results were interpreted as "evidence against resorption of pentoses through the kidney tubules by phosphorylation," but it may well be that phosphorylation of pentoses requires a more complicated system than that for glucose, and that the necessary co-factors were not supplied.

*Action of agents which decrease efficiency of energy utilization.*----

A number of agents exist which have the property, when added in appropriate concentrations to various tissues, of increasing the rate of exergonic reactions while simultaneously decreasing the energy utilizing functions; the result is of course an increased heat production. The action of several of these agents is summarized in Table I. Hotchkiss (131) recently found that dinitrophenol (DNP) prevented phos-

TABLE I  
AGENTS WHICH AFFECT THE COUPLING OF OXIDATION-REDUCTION  
REACTIONS WITH ENERGY UTILIZATION

Agent	Process stimulated	Process inhibited	Reference
Dinitrophenol (DNP)	Respiration and glycolysis	Maintenance of phosphocreatine	126
DNP .....	Respiration and glycolysis	Assimilation	127
DNP .....	Respiration and glycolysis	Sperm motility	128
Azide .....	Yeast fermentation	Assimilation	129
Chloral hydrate or chlorotone .....	Respiration	Assimilation and luminescence	130
Gramicidin .....	Respiration	Assimilation, P uptake	131
Dysentery toxin .....	Hydrolysis of ATP		132

phate uptake by bakers' yeast respiring in glucose and that the bacteriostatic action of gramicidin may also be related to its effect in preventing phosphate uptake. The concept of high energy phosphate as the intermediate energy carrier between oxido-reductions and energy utilization, and as the mechanism controlling the rate of oxidation and glycolysis (133) invites the hypothesis that those agents which speed up metabolism and at the same time decrease the energy available for work or assimilation act by allowing oxidations to occur without phosphorylation<sup>3</sup> or actually cause dephosphorylation of high energy phosphate. Early evidence for this was provided by the finding that DNP

<sup>3</sup> Compare the effect of arsenate on the oxidation of triosephosphate.

caused a decrease of phosphocreatine in frog muscle (126). DNP was also found to increase the rate of hydrolysis of ATP added to minced rat muscle (134).

While the results of Hotchkiss mark a new advance in the study of the action of gramicidin, it seems they cannot be explained on the basis that gramicidin inhibits phosphate transfer mechanisms (135). Were this the case, the phosphate acceptors would accumulate in the phosphorylated form and the oxidative mechanisms which are obligatorily coupled with phosphorylation would be retarded. A more plausible explanation would be that gramicidin and certain of these other agents allow an exergonic step to occur without phosphorylation or that they catalyze the anomalous hydrolysis of a phosphate ester. This would account for the acceleration of oxidative and glycolytic processes and the failure of endergonic syntheses or work performance.

The mold pigment, *Rubrofusarin*, apparently increases the efficiency of coupling oxidations with assimilation for Mull & Nord (136) found that addition of a solution of the pigment to an unpigmented mold increased the mat weight by 40 per cent while causing a slight decrease in dehydrogenation.

#### INTEGRATION OF FAT AND CARBOHYDRATE OXIDATION

*The isocitric (tricarboxylic) acid cycle.*—Research has continued to augment the evidence supporting the concept of the majority of carbohydrate oxidation going through the isocitric acid cycle, and recent reports from a number of sources (137 to 141) indicate that fat metabolism, also, may follow a similar pathway. The newer concepts, permitted by recent accomplishments in a number of divergent fields, are embodied in the schematic diagram in Figure 1 and will be discussed individually.

The key substance in the cycle is the two-carbon compound which condenses with oxaloacetate and, in accordance with the most recent evidence, is here designated as an "activated" acetyl radical. Acetyl phosphate is now established as the product of pyruvate dissimilation in a number of bacterial preparations (115, 116, 117) and it is also the substance that undergoes further oxidation to carbon dioxide and water (142). It seems possible that acetyl phosphate is also the key intermediate in fat metabolism following Lehninger's finding that acyl phosphates rather than fatty acids are attacked by liver enzymes (80) and recent confirming evidence that fat oxidation involves successive removal of two-carbon fragments which can condense to yield



pears predominantly in the carboxylic part of the acetoacetate (148) would be expected if acetyl phosphate were the activated form since the direct phosphorylation of acetate by ATP is less easily performed than the production of acetyl phosphate from carbohydrate.

While ATP is necessary to phosphorylate the carboxyl groups of fatty acids before the chain is oxidized (80, 81), it is probable that the  $\beta$  oxidation of the fatty acid leaves an acyl phosphate as a residue rather than the free acid with a chain shortened by two carbon atoms. Thus once the reaction is primed, no additional expenditure of ATP phosphate bond energy would be required for the continued oxidation and the further oxidation of the fat would, as has been shown (113), result in the uptake of inorganic phosphorus and the support of energy-utilizing functions. An inviting hypothesis to account for the fact that no appreciable quantities of fatty acids of intermediate chain length can be recovered from tissues would be the assumption that the affinity of the fat oxidizing enzyme for the acyl phosphates is sufficiently great to permit the desmolysis of one chain completely before it attacks a new one [see also Weinhouse, Medes & Floyd (86)]. The level of ATP in a tissue may well control the relative amounts of fat and carbohydrate oxidized and the preferential utilization of carbohydrate under conditions of great energy expenditures may be the result of a decrease in the ratio ATP/adenylic acid where the ATP is being utilized during muscle contraction. The "priming" phosphorylations of carbohydrates produce ester phosphate bonds with only *ca.* 3000 calories of free energy per mole and should proceed at a lower ATP "potential" than the production of acyl phosphate which contains an 11,000 calorie bond.

It has been established that the tricarboxylic acid actually oxidized by the tissue enzymes is isocitric acid but the only definite statement that can be made about the primary condensation product of oxaloacetate and the acetyl residue is that it is not a symmetrical compound like citric acid (149, 150). Early proponents of the cycle frequently included the pyruvate-oxaloacetate addition product, 2-keto, 4-hydroxy, 4-carboxyadipic acid in their diagrams of the cycle. Martius, whose fundamental work established many of the reactions of the cycle, has now synthesized the 1,4-lactone of this compound, devised a method for its determination, and studied its possible metabolic role (151). Incubation of hog heart, rabbit kidney, and liver pulp with pyruvate and oxaloacetate under anaerobic conditions resulted in almost theoretical quantities of citric acid with no accumulation of a

7-carbon compound. When synthetic 2-keto, 4-hydroxy adipic acid was added to these tissue preparations, no citrate was formed and the original compound could be recovered quantitatively unchanged. It can thus be ruled out as the primary condensation product between oxaloacetate and the carbon chain of pyruvate.

While *cis*aconitate remains the most plausible precursor of isocitrate, its occurrence as an essential step in the cycle is difficult to reconcile with the findings of Evans & Slotin (150). These workers reported  $\alpha$ -ketoglutarate production from pyruvate and labeled bicarbonate by preparations of pigeon liver which did not allow added citrate to dilute the radioactive carbon in the product. From what we know of the dynamic equilibrium of body constituents it seems likely that added citrate would have entered the cycle and diluted the product if aconitase were active in the liver preparation. Tests for aconitase were not reported by Evans & Slotin (150).

The report of Breusch (137) that acetoacetate condenses with oxaloacetate to form citrate has been criticized by Weil-Malherbe (152) and Krebs & Eggleston (153).

*Metabolite interconversion.*—The diagrammatic presentation of lactate and  $\beta$ -hydroxybutyrate as reduction products of pyruvate and acetoacetate respectively and not as intermediates in the oxidation of propionate and butyrate is in agreement with the earlier reports of Friedmann & Maase (154) and Jowett & Quastel (155) that  $\beta$ -hydroxybutyrate is not an intermediate in the oxidation of butyrate, and the recent finding of Barker & Lipmann (156) that propionate can be oxidized to pyruvate by enzyme preparations from *Propionibacterium pentosaceum* in the presence of concentrations of fluoride which completely inhibit lactate oxidation. While vinylacetate may be an intermediate in the oxidation of butyrate (84), the homologous acrylate does not seem to be the intermediate in propionate oxidation (156).

The mechanism of phosphopyruvate resynthesis from unphosphorylated carbohydrate residues remains undisclosed. The fact that lactate and pyruvate oxidation did not yield phosphopyruvate in the experiments of Leloir & Munoz (79) while succinate, fumarate, and citrate oxidation did, might appear to support the frequently postulated formation of phosphoenoloxaloacetate which may be decarboxylated to phosphopyruvate. Two points militate against this however. The fact that adenylic acid was necessary for the formation of phosphopyruvate from succinate, fumarate, or citrate indicates the possibility of phosphate transfer rather than direct uptake of inorganic

phosphate into the carbon chain which is eventually to yield phosphopyruvate. Furthermore, malonate inhibited the formation of phosphopyruvate from succinate, fumarate, and citrate to approximately the same extent. It has not yet been ruled out that the superiority of the four-carbon acids over pyruvate and lactate in the formation of phosphopyruvate is the result of their catalytic activity rather than their superiority as a carbon chain to be phosphorylated.

*Hydrogen transport.*—The Szent-György scheme of hydrogen transport has been proved non-essential for the oxidation of triose phosphate (157),  $\alpha$ -ketoglutarate (110), and of alcohol and acetaldehyde to acetic acid (138). However, Barron (158) considers the scheme an important pathway for the transport of hydrogen and proposes that all oxaloacetate arises from carbon dioxide addition to pyruvate, none from oxidation of malate. No provision was made for the oxidative removal of four-carbon dicarboxylic acid which would rapidly accumulate if a mole of oxaloacetate were formed for every four atoms of hydrogen removed from metabolites.

#### CARBON DIOXIDE FIXATION AND REDUCTION

The biochemistry of the chemo-autotrophic bacteria was the subject of a review by van Niel (159).

*Photosynthesis.*—The “mysteries” of the process of photosynthesis are gradually being revealed as a series of enzymatically catalyzed reactions, which are common to many tissues, coupled with the energy-yielding “photochemical reaction.” Progress in this interesting and significant field has been the result of attempts to separate and define the various reactions. Photosynthesis and the carbon dioxide reduction reactions in algae were authoritatively reviewed by Gaffron (35) during the year and only the more recent developments will be considered here.

Notable examples of the separation of the various phases of photosynthesis are (a) the demonstration by Gaffron [see (35)] that certain algae adapted to use hydrogen gas can fix and reduce carbon dioxide without oxygen liberation, (b) the finding, by Hill & Scarisbrick (160), that isolated chloroplasts which have no ability to reduce carbon dioxide will produce oxygen during the photoreduction of ferric salts, and (c) the recent separation, in *Chlorella*, of oxygen liberation from carbon dioxide reduction as reported by Fan, Stauffer & Umbreit (161). The latter workers were able to show that, in the

absence of carbon dioxide, the addition of a number of reducible substances to suspensions of *Chlorella* allowed production of oxygen during illumination. Detailed studies were made using benzaldehyde; it was shown that this substance did not produce carbon dioxide and that illumination in the absence of benzaldehyde or other reducing substances did not result in oxygen production.

The theory that "the function of light energy in photosynthesis is the formation of 'energy rich' phosphate bonds" was proposed by Emerson, Stauffer & Umbreit (162) who presented evidence that, in *Chlorella*, the distribution of phosphate compounds was markedly different after irradiation either in the presence or absence of carbon dioxide from that in control suspensions kept in the dark. Accumulations of energy rich phosphate during irradiation in the absence of carbon dioxide with subsequent utilization of the energy for carbon dioxide reduction in the dark could not be demonstrated with *Chlorella* (162) as it was clearly done with *Thiobacillus thiooxidans* (163, 164) in which sulfur oxidation rather than radiant energy is the source of energy. Nor could the phosphate compounds of *Chlorella* (162) be characterized as were those of the chemo-autotrophic organism (165).

If high energy phosphate does prove to be the primary form of energy fixed in the photochemical reaction, it may be utilized for carbon dioxide reduction, as Ruben suggested (166), by the formation of carboxyl phosphate in which the opposing resonance decreases the stability and hence renders the carboxyl group more easily reduced.

The isolation of several strains of non-sulfur purple photosynthetic bacteria adapted to the oxidation of various alcohols was described by Foster (167). In these organisms the hydrogen required for the photoreduction of carbon dioxide is obtained from the dehydrogenation of the alcohols.

*Carbon dioxide fixation in heterotrophic organisms.*—While in the living heterotroph the metabolic reactions proceed toward decarboxylation of the metabolites, there is no reason to doubt that in the steady state all of these reactions are reversible. The demonstrated equilibrium between pyruvate and acetyl phosphate plus formate (118) was discussed above. The difficulty in demonstrating the reversibility of these enzyme-catalyzed reactions lies in obtaining enzyme preparations which do not catalyze the hydrolysis of the unstable energy rich compounds required to reverse the reaction. Carbon dioxide fixation is probably of greatest importance to the higher animals in providing

a means for the production of substances required for catalysis of intermediate metabolism. Such endergonic reactions are probably called upon only when the normal production of these substances by exergonic reactions from ingested foodstuffs does not proceed at a rate sufficient to meet the organism's demands. This is not to say that carbon dioxide is not in equilibrium with the metabolites in the cell, carboxylations being energetically balanced with decarboxylations, but does imply that, as yet, no satisfactory evidence has been offered that carbon dioxide fixation is an essentially exclusive step in the formation of any body constituent in the higher animals. In fact, the finding of Evans, Vennesland & Slotin (168) that carbon dioxide is fixed in pyruvate, possibly through the Wood & Werkman reaction followed by an enol shift and subsequent decarboxylation of the oxaloacetate, may make necessary a reevaluation of previous conclusions regarding the necessity of tissue bicarbonate for the formation of glycogen (169). The results indicating that one sixth of the newly deposited glycogen after glucose, lactate, or pyruvate feeding originated in tissue bicarbonate may be a reflection of the above described equilibrium rather than of the necessity for a four-carbon intermediate in the production of phosphopyruvate from pyruvate.

The decarboxylation of oxaloacetate by extracts of *E. coli* required the addition of manganese salts but not cocarboxylase or inorganic phosphate (171). The enzyme preparation catalyzed the formation of oxaloacetate from fumarate, malate, or succinate under aerobic conditions and also produced, under anaerobic conditions, oxaloacetate, or a chemically similar substance, from pyruvate and bicarbonate. Maximal carbon dioxide utilization occurred in the presence of hydrogen gas but under these conditions no oxaloacetate accumulated. Kalnitsky & Werkman (171) carefully point out [contrary to the misinterpretation of Evans (170, p. 282)] that the simultaneous formation in the extract of acetyl phosphate from pyruvate is a logical source of the energy required for the endergonic production of oxaloacetate. The possible occurrence of the Wood & Werkman reaction in rat muscle extracts was indicated (121).

The finding that *Clostridium thermoaceticum* ferments glucose with the unusual production of more than two moles of acetate per mole of glucose (172) suggested that either the glucose chain was split into three two-carbon compounds or that the usual triose split was followed by carbon dioxide production and subsequent utilization. The latter seems the more probable since Barker (173) found that the organism



produces more than two molecules of acetate per molecule of xylose and more than one molecule per molecule of pyruvate fermented.

The effect of carbon dioxide tension on the metabolism of brain tissue (174) and yeast (175) was reported.

### METHODS

A new book by Umbreit, Burris & Stauffer (176) contains excellent descriptions of procedures for carrying out several types of manometric respiration experiments. Included also are methods for the preparation of many compounds which are intermediates in metabolism and for the quantitative determination of intermediates and end products in the amounts usually produced by tissues in a single manometer flask.

Special apparatus for measuring tissue respiration under several atmospheres of pressure were designed (177, 178). Fuhrman & Field (179) emphasized the importance of keeping tissues at a low temperature during the preparation of slices for respiration studies. Schales (180) presented a simplified procedure for the calibration of manometric apparatus.

### OXIDATION-REDUCTION POTENTIALS

The oxidation-reduction potentials of a number of substituted naphthoquinones were determined by Wallenfels & Mohle (181). Ryk-lan & Schmidt (182) studied the oxidation potentials of a number of sulfhydryl systems including these which are of direct biological importance: cysteine-cystine, glutathione, and ergothioneine. The free energies and equilibrium constants of the systems were calculated.

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# ENZYMES THAT HYDROLYZE THE CARBON-NITROGEN BOND: PROTEINASES, PEPTIDASES, AND AMIDASES

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## INTRODUCTION

This review follows the well-established pattern of previous years (1, 2), and seeks to cover recent advances in the fields of the proteolytic enzymes and amidases. Some of the papers reviewed are from foreign journals dating back to the first years of the war that were unavailable until now.

Considerable progress has been made in the purification of certain members of the group under discussion. New sources of proteolytic enzymes have been discovered, particularly in the higher plants. The relation of oxidation-reduction to the activity of the papain-like enzymes, and of divalent metallic cations to the activity of arginase and many of the peptidases, has been subjected to closer scrutiny. Advances have been made in the clarification of the specificities of that complex and tangled group of enzymes, the peptidases, although much still remains to be uncovered.

*Nomenclature.*—In the estimation of the reviewers, it is unfortunate that there is a tendency to name different groups of proteinases after some particular group representative, because such a terminology offers no specific clue to the property or properties that serve as a basis of classification. For example, the proteolytic enzymes that are activated by reducing agents are now generally called papainases, after one member of this group. Another example is the loose tendency to apply the terms "pepsin" or "pepsinase" to any enzymatic material whose optimum proteolytic activity is at a distinctly acid pH and "trypsin" or "trypsinase" to material with its optimum activity at an alkaline pH. Possibly the more descriptive terms "acidoproteinase," "neutroproteinase," and "basoproteinase" would be better to use for indicating the relationship between optimum activity and pH. In the more desirable type of classification based on the specificity toward

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certain synthetic substrates, proposed by Bergmann and co-workers (3), enzymes having the same specificities as pepsin and trypsin take the name pepsinase or trypsinase. This, unfortunately, has led to such unsatisfactory terms as "papain-trypsinase" (4). The reviewers urge that, in the interest of clarity, the names devised to designate different classes of proteinases be as descriptive as possible of the nature and mode of action of the enzymes.

## PROTEINASES (ENDOPEPTIDASES)

### GENERAL AND THEORETICAL

*Protein hydrolysis products.*—Although Bergmann and his associates have contributed valuable information through their pioneer studies with synthetic peptide substrates, there is still a great lack of knowledge as to the exact types of peptide linkages hydrolyzed in proteins by various proteolytic enzymes. That proteinases such as pepsin, trypsin, and chymotrypsin act differently on a given protein was shown by Northrop (5). Recent evidence (6, 7) indicates that purified proteinases do not hydrolyze the terminal peptide bonds of the polypeptide chains in proteins to any great extent, since only small amounts of free amino acids are liberated. The action of peptidases, however, consists chiefly in the splitting of terminal, rather than more centrally located peptide bonds in peptides (3, 7, 8).

Certain of the differences between the action of crude "trypsin" and crystalline trypsin on proteins were indicated in a paper by Kotel'nikova (9). This investigator found that, whereas tyrosine and tryptophane were liberated quantitatively from casein, fibrin, and edestin by impure trypsin preparations, the percentage of the total tyrosine liberated by crystalline trypsin was only 12 per cent for casein, 24 per cent for fibrin, and 14 per cent for edestin. Free tryptophane was obtained only from fibrin when crystalline trypsin was used, and no ammonia was formed from any of the proteins.

The peptides which result from the action of a proteinase on a protein very likely constitute a highly complex mixture, difficult to resolve. The isolation of individual peptides from such mixtures, and the determination of their amino acid make-up, would probably throw much light on the whole problem of protein structure, as well as on the mode of action of the enzyme. However, Syngé (10), in reviewing recent work on partial hydrolysis products of proteins, points out that enzymic hydrolysis, unlike hydrolysis by acid, may be compli-

cated by simultaneous synthetic reactions. Bergmann (3) likewise notes this possibility in discussing coupled reactions induced by enzymes.

While recognizing the possibility that enzymic digests may represent the resultant of hydrolytic and synthetic reactions, a recent study by Winnick (7), employing crystalline proteases, presents several types of information relative to the partial hydrolysis products of casein. The use of enzymes avoided the danger of racemization or partial destruction of amino acids during the hydrolysis, and the high activity of the enzymes employed made it possible to use very small amounts of the latter relative to the amounts of substrates; of the order of one to several hundred parts. In this study, the method described by Van Slyke *et al.* (6) was used to estimate the number of amino acid residues in the average nonprotein molecule. It was found that the latter was approximately a pentapeptide following the action of chymotrypsin, ficin, or papain, and a heptapeptide following the action of pepsin or trypsin, on casein. These results were then compared with average molecular weight values, determined by cryoscopic measurements, on the same nonprotein digests. The freezing point determinations, made after the removal of inorganic electrolytes by electro-dialysis, indicated average molecular weights ranging from 450 to 600. It seems of interest that the initial nonprotein products from short periods of digestion did not differ significantly from those of prolonged proteinase action, as judged from specific optical rotations and ratios of amino to total nitrogen. This observation appears to support the "explosion" hypothesis of proteolysis, proposed by Tiselius & Eriks-son-Quensel (11), which states that proteinase action consists in the very rapid splitting of relatively few protein molecules in each time interval, rather than in a general slow cleavage of all of the protein molecules simultaneously.

*Methods of measuring protease activity.*—The complexity of proteins and of the process of proteolysis has led to development of a great variety of methods for the measurement of the activity of proteinases. Many workers continue to employ methods of assay which do not distinguish between the primary stage of proteolysis and the later stages, which are more indicative of peptidase action. Probably the best measure of proteinase activity is given by the estimation of the nonprotein nitrogen (12) or the phenol color value (13) of the split products soluble in dilute trichloroacetic acid solution. The phenol color method employs a more reproducible protein substrate, de-

natured hemoglobin, but a disadvantage is that certain reducing agents, such as cysteine, interfere with the colorimetric measurements.

A simple and fairly accurate measure of the nonprotein formed in digestion mixtures can be obtained by observing the optical rotation of the trichloroacetic acid filtrates, with casein as the substrate (14).

The changes in viscosity, contraction of volume, or increase in amino or carboxyl groups, generally are insufficient for distinguishing between proteinase and peptidase action when proteins are employed as substrates. Use of gelatin as a substrate is open to certain criticism. Lennox (15), in his study of the action of trypsin, papain, euphorbain, and a mold proteinase on gelatin, found that there was no constant relation between the time required for a given decrease in viscosity and the enzyme concentration. Anson (16) observed that purified cathepsin (free of peptidase) does not cause a detectable increase in formol titration when added to gelatin at pH 3.5.

An ingenious method of measuring proteolytic activity, introduced by Linderstrøm-Lang & Jacobsen (17), is based on the determination of the contraction in volume of the digestion mixture (2). This volume change is due mainly to the electrostriction produced by the increase in electric charges rather than the water added to the peptide bond. Thus, when glycylglycine is hydrolyzed (at pH 6) two new electrical charges are formed which cause an increased compression of the surrounding water. Proof for this is offered by Jacobsen (18) who found that the splitting of benzoyl-*dl*-argininamide by trypsin produced a volume contraction of  $15.6 \pm 0.8$  cc. per mole and of benzoyl-*L*-tyrosylglycinamide by chymotrypsin  $13.4 \pm 0.2$  cc. per mole of split peptide. These approximate the calculated values.

When synthetic peptides are employed as substrates, the hydrolytic activity of a proteinase or a peptidase can be expressed in terms of the proteolytic coefficient, *C* (19). This coefficient is the ratio of the first order velocity constant, *K*, to the enzyme concentration, *E*, expressed in mg. of nitrogen per cc. of test solution. The hydrolysis is usually measured by titration of the liberated carboxyl groups.

Other recent methods which may be of value, particularly in peptidase assays, are the copper method of Pope & Stevens (20), which measures amino nitrogen, and the ninhydrin-carbon dioxide method of Van Slyke and associates (6), which distinguishes between free amino acids and peptides.

*Use of impure proteases.*—The use of impure enzyme preparations in studies which require pure enzymes for critical results is still too

common. Examples are the study by Kauffman & Urbain (21) of the inactivation of trypsin and papain solutions by ultraviolet radiation, and a paper by Kleczkowski (22) on the combination of viruses with pepsin and trypsin. Commercial "trypsin" or pancreatin is, of course, a mixture of proteases, and contains a large proportion of inert material. The preparation of crystalline proteases, particularly those of animal origin, offers no great difficulty. For example, Plentl & Page (23) prepared crystalline pepsin, trypsin, chymotrypsin, and carboxypeptidase, and recrystallized these enzymes three times for their study of the structure of angiotonin. In the light of recent progress in protein and enzyme chemistry, it seems doubtful that many of the studies with impure proteases and ill-defined substrates will have lasting value.

#### "SPECIFIC" PROTEASES

Information has accumulated in recent years on a number of physiologically active proteins which appear to function in a proteolytic manner, and generally act on specific substrates. The best known of these catalysts are thrombin (and prothrombin), thromboplastin (thrombokinase), and renin.

*Blood clotting system.*—The action of thromboplastin on prothrombin is analogous to the activation of trypsinogen by enterokinase, while the action of thrombin on fibrinogen appears to involve proteolytic changes.

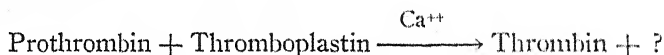
Protein preparations from lung with high thromboplastin potency have been prepared in several laboratories (24, 25). According to Chargaff, Bendich & Cohen (24), highly purified beef lung thromboplastin contains both ribose nucleic acid and acetal phosphatides in chemical combination with a specific protein. The lack of proteolytic activity on the part of the thromboplastin, as shown by its inability to attack gelatin, is of interest because of the contrast to trypsin, which also has thromboplastic activity. The phosphatide of thromboplastin is commonly considered to be cephalin. Chargaff (26), however, is of the opinion that the phospholipid component cannot yet be identified with any of the known phosphatides.

Ferguson (27), in a new theory of blood coagulation, has suggested that in the blood the trypsinase of plasma, a phosphatide, and calcium ions act as thromboplastic agents for the conversion of prothrombin to thrombin. This reaction is kept in check by the trypsin inhibitors normally present in blood. Dyckerhoff & Torres (28) have concluded that prothrombin exists as a thrombin-inhibitor complex

and activation consists in the removal of the inhibitor by adsorption on thromboplastin. The concept of proteolytic action was doubted by these latter investigators because they observed that thromboplastin could be boiled for a short time without loss of activity. The evidence seems inadequate in view of the similar heat stability of the trypsin enzymes.

From experiments with blood plasma, Quick (29) concluded that prothrombin is composed of two separable components combined through calcium. One of the two components is apparently destructively oxidized by air and the other may be removed from oxalated plasma by adsorption with aluminum hydroxide. The second component disappears from the plasma of animals poisoned with dicumarol.

Loomis & Seegers (30) have challenged the views that calcium is an integral component of prothrombin or thrombin or that spontaneous activation of prothrombin in concentrated ammonium sulfate is due to this salt (31). Of the ions tested, only calcium and strontium aided the conversion of prothrombin to thrombin by thromboplastin. Calcium was by far the more effective. No other ions showed any significant activity and in high concentrations they all inhibited the activating effect of calcium. Loomis & Seegers concluded that calcium acts as a catalyst for the reaction:



Highly purified prothrombin and thrombin have now been prepared by a number of investigators by different procedures (31 to 34).

The procedure of Seegers and co-workers (32, 33) involves the adsorption of prothrombin on magnesium hydroxide, elution by decomposing the magnesium oxide with carbon dioxide, and salt fractionation at 0° with ammonium sulfate.

Seegers & McGinty (33) isolated snow white preparations of thrombin having a potency of 12,000 units<sup>2</sup> per mg. of nitrogen by repeatedly adsorbing the transformed thrombin on inert denatured serum protein at pH 5.0. By means of solubility measurements it was estimated that this product contained less than 10 per cent of inert material but that it was composed of two active components which differ distinctly in solubility and probably in potency. Milstone (31) has prepared purified thrombin having 100 to 175 times the activity of

<sup>2</sup> The unit of thrombin is defined as the amount of activity required to cause clotting of 1 cc. of standardized fibrinogen solution at 28° in 15 seconds.

whole plasma by repeated fractionation with saturated ammonium sulfate. Thrombin is obtained among the products in the system of fractionation of blood plasma proteins developed by the Harvard Medical School group (34).

Seegers (35) found that solutions of thrombin could be protected against loss of activity by saturated solutions of a large number of carbohydrates and their derivatives, or by glycerol solutions. Prothrombin was stabilized to a lesser degree by these solutions.

Components in blood serum reported to be concerned in inhibiting blood coagulation by Astrup & Darling (36) are antithrombin, a non-dialyzable albumin, and thrombin-coinhibitor, which is said to be necessary for the formation of an antithrombin with heparin.

*The renal vasopressor system.*—The literature through 1943 on the enzymatic aspects of the blood pressure mechanism has been reviewed by Mann & Lutwak-Mann (37). The present concept of the processes involved is that renin, a specific proteinase produced in the kidney and discharged into the blood stream, reacts with a globulin fraction from the blood, i.e., renin substrate or hypertensinogen (38, 39), to produce a pressor hormone (angiotonin or hypertensin), which appears to be a polypeptide (23). The latter is believed to be inactivated by another enzyme-like substance, present in kidney and other tissues and not yet well characterized, which has been named angiotonase or hypertensinase. In blood serum, an appreciable quantity of angiotonase has been demonstrated in the albumin fraction, but none could be found in the globulins (38). The formation and destruction of angiotonin has been shown to consist of two consecutive first order reactions (40). To the above may be added antirenin, a thermolabile pseudo-globulin produced in rabbit, guinea pig, or dog plasma by the injection of renin from different species (41).

Plentl & Page offer the following evidence in support of the enzymatic nature of renin and of angiotonin formation (42): (a) Renin is a heat-labile protein, (b) the rate of formation of angiotonin is proportional to the renin concentration (at low renin concentrations), (c) for a given amount of renin substrate the maximum yield of angiotonin cannot be surpassed by varying the renin concentration over a wide range, and (d) the formation of angiotonin can be represented by a first order reaction equation.

Plentl & Page (43), in a recent study of the specificity of renin, have tested their pressor preparations on a variety of synthetic substrates. The resulting hydrolyses indicated the presence of carboxy-

peptidases, pepsinases, trypsinases, and aminopeptidases in the renin preparations. It had been previously shown that pepsin could be substituted for renin in the preparation of an angiotonin-like substance called pepsitensin (44), and it was implied that pepsin (or kidney pepsinase) and renin were homospecific<sup>3</sup> in their action. However, from certain evidence, including fractional denaturation, and the fact that pepsin inactivates angiotonin while renin can be prepared free of angiotonase activity, Plentl & Page have concluded that renin is not the same chemical entity as kidney pepsinase. But since homospecificity was still not excluded, they compared the action of renin and crystalline pepsin on two test substrates, carbobenzoxy-L-glutamyl-L-tyrosine and renin substrate (hypertensinogen). The ratio of the proteolytic coefficients of the renin and pepsin on renin substrate was found to be many times greater than the corresponding ratio on the synthetic peptide, indicating heterospecificity. Accordingly, it seemed improbable that the kidney pepsinase was homospecific with renin.

A comparison of the inactivation of angiotonin and pepsitensin solutions of equal pressor strength by crystalline pepsin yielded differences in proteolytic coefficients and optimum pH values in the two cases indicating that these hormones are not identical. The conclusion was drawn that both substances are probably polypeptides that differ in the number rather than in the nature of the amino acid residues of which they are composed (43).

*Miscellaneous.*—Other biologically active substances associated with proteolytic activity are hemolysin, a crystalline protein from snake venom, which can be inactivated by oxidizing agents and reactivated by hydrogen sulfide and potassium cyanide, and whose antigenic properties have been studied (45); fibrinolysin of  $\beta$ -hemolytic streptococci, which is probably a proteolytic enzyme, and which is completely inhibited by either crystalline trypsin inhibitor of pancreas or anti-trypsin of soybean origin (46); a crystalline albumin from horse diphtheria anti-serum, which, after activation by trypsin, has strong specific enzyme action against diphtheria serum (47); and necrosin, a euglobulin found in inflammatory exudates, which may be a proteolytic enzyme, and which is associated with a pyrogenic factor, pyrexin

<sup>3</sup> This term, as defined by Bergmann (3) and employed by Plentl & Page, applies to enzymes which have the same proteolytic quotients for a given pair of substrates. The proteolytic quotient is the ratio of the proteolytic coefficients (defined in a preceding section of this review) of an enzyme on two different substrates.

(48). The latter may be the product of the action of necrosin on a substrate in injured cells.

#### ANIMAL PROTEINASES

*Rennin*.—The successful crystallization of rennin, the milk coagulating enzyme of the stomach, has been reported by Hankinson (49) and by Berridge (50). Hankinson's procedure consists of repeated precipitation of rennin from commercial rennet extract by adjusting the pH to 5.0, and salting out with saturated sodium chloride. After the fourth precipitation the rennin is dialyzed until free from sodium chloride, the dialyzed material is diluted to 0.05 per cent solids concentration, adjusted to pH 5.7 to 6.0, and filtered. Crystallization is accomplished by the slow addition of 0.1 *N* hydrochloric acid with stirring to the point of turbidity (pH 4.5). Success in obtaining crystalline rennin depends upon sufficient preliminary purification, a solids content not greater than 0.05 per cent, the slow addition of acid, and maintenance of the temperature during crystallization between 20 to 25°. Crystalline rennin has needle-shaped crystals, a milk clotting activity of eighteen to twenty-one times that of commercial rennet per mg. of nitrogen, and its isoelectric point is between pH 4.45 and 4.65.

The rennin obtained by Berridge by repeated fractional precipitation with saturated magnesium sulfate consisted of flat crystalline plates. Berridge found the solubility to be independent of the amount of solid phase present, although the nitrogen content of his rennin was only 13 per cent, in contrast to 15 per cent obtained in Hankinson's preparation.

*Pepsin*.—An important note by Harington & Rivers (51) relates to the action of crystalline pepsin on both acylated and nonacylated (free) tyrosine-cystine (and cysteine) peptides. While tyrosylcysteine, tyrosylcystine, cysteinyltyrosine, and cystinyltyrosine were hydrolyzed to a somewhat lesser extent than their corresponding *N*-carboxybenzyloxy derivatives, a significant hydrolysis (ranging from 0 to 31 per cent) of the free peptides did occur at pH's of 1.8 and 4.0. The results were not in accord with the generalization of Bergmann regarding the necessity of more than one free carboxyl group (in the vicinity of the labile peptide bond) and the inhibitory action of free amino groups, in peptic substrates. Hitherto the only synthetic substrates available for pepsin were those in which an amino group of an aromatic amino acid was combined with the  $\alpha$ -carboxyl of an acylated



glutamic acid. Harington & Rivers also point out that of their peptides, those with free thiol groups showed a greater susceptibility to peptic hydrolysis, and suggest that this fact may be related to the increase in digestibility and appearance of sulfhydryl groups, which result upon the denaturation of many proteins.

One point which impressed the reviewers in connection with the preceding paper, and specificity studies in general, was the apparent difficulty of distinguishing between qualitative and quantitative differences in the hydrolyzability of certain peptides. Unless equilibria are involved, the degrees of hydrolysis often seem small for periods as prolonged as twenty-four or forty-eight hours. Lastly, while Bergmann & Fruton (52) have maintained that pepsin substrates must contain at least two free carboxyl groups for optimal hydrolysis, they find that some hydrolysis still occurs when one of the carboxyls is masked by amide formation. For example, carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide is split to the extent of 15 per cent in forty-eight hours at pH 4.0.

The question of a protein-free pepsin has been raised again by Albers, Schneider & Pohl (53). These investigators obtained a pepsin fraction which was heat- and acid-resistant, soluble in 70 per cent ethanol, and not precipitated by sulfosalicylic acid or by basic lead acetate. This fraction, called "pepsidin," could be ultrafiltered through collodion membranes, and had a higher tryptophane content than Northrop's crystalline pepsin. Its optimum action on casein was at pH 7 to 7.5, but after mild oxidation with hydrogen peroxide it showed a steep optimum at pH 2 and another, much lower maximum, at pH 7.6 to 8.0. As little as 30  $\gamma$  of concentrated pepsidin showed marked proteolytic activity, but its removal from pepsin preparations did not greatly alter the residual activity from that of the initial pepsin. Pepsidin, unlike pepsin, attacks peptones. Albers, Schneider & Pohl regard their pepsidin as a polypeptide "coproteinase" which is normally combined with a protein carrier, but which can digest casein in the absence of the carrier. The authors are in doubt as to whether pepsidin is admixed with pepsin in gastric juice, or whether it should be considered a part of the pepsin or trypsin molecule.

It is difficult to assess the significance of these observations. In the light of the clear-cut, painstaking work of Northrop and his associates the experimental data appear inadequate to support the conclusions drawn. It is not improbable that pepsidin is a fraction containing mainly nonpepsin proteinase and also peptidases. Cathepsin and erep-

sin have been observed in gastric mucosa (54) and cathepsin in gastric juice (55).

In connection with the above, the preparation by Borgstrom & Koch (56) of amorphous pepsin of higher enzymatic activity than crystalline pepsin from commercial and even from crystalline pepsin is of interest.

In connection with the work on pepsidin, Albers, Schneider & Pohl (57) made the observation that phenyl hydrazine after mild oxidation with hydrogen peroxide will hydrolyze casein with optima at pH 2 and 6.5 and peptone with optima at pH 2.8 and 7.8.

Hind (58) has noted that pepsin added to fibrinogen will inhibit the precipitation of the latter protein by ammonium sulfate (one gm. crude pepsin will protect 50 gm. fibrinogen) and that fresh blood serum contains a substance which counteracts the inhibiting effect of pepsin on the salting out of fibrinogen.

*General.*—Analysis of purified trypsin, chymotrypsin, and rennin (together with a number of other proteins) has indicated that these enzymes contain no significant amounts of any of the B vitamins (59), a finding which agrees, of course, with the evidence that proteinases contain no other groups than protein in their molecules.

The interesting question as to whether pepsin and trypsin can combine only with substrates, or whether combination can also occur with proteins not hydrolyzed by the enzymes, was considered by Kleczkowski (22). Previous work by Stanley had shown that tobacco mosaic virus solutions lost their infectivity when treated with trypsin at pH values at which the enzyme was not active. In studying the combination of potato X virus and tobacco mosaic with pepsin and trypsin, Kleczkowski found that pepsin combined with potato X virus, which it also hydrolyzed, but not with tobacco mosaic virus, which resisted digestion. However, when the latter virus was denatured by heat, it combined with, and was digested by pepsin. With trypsin the situation was reversed, in that this enzyme combined more readily with tobacco mosaic virus, which is not a substrate, than with potato X virus, which is digestible.

The possibility of using ethyl alcohol as an aid in preventing putrefaction during the prolonged enzymatic digestion of serum and other proteins is suggested by the observation that 70 to 80 per cent of the potential amino groups of serum proteins were liberated upon prolonged digestion with trypsin (Difco brand) in 10 to 30 per cent ethanol solution (60). While concentrations of alcohol greater than

30 per cent were inhibitory, some hydrolysis occurred even in 60 per cent ethanol.

In a study dealing with cathepsin of liver, Schöffner & Truelle (61) have sought to demonstrate the presence of both a hemoglobin-splitting enzyme (HE) and a gelatin-splitting enzyme (GE). The HE was obtained free of GE by a process which included destruction of the latter by alkali, while the GE was isolated from the starting material by an autolytic process which destroyed the HE. Whereas the GE was activated by cysteine and inactivated by iodoacetate, the HE was not affected by these reagents. The GE had an optimum pH of 4.3 to 4.5, while the HE had the same optimum pH (3.5) on hemoglobin as Anson's purified cathepsin (13). Although the GE failed to digest serum albumin or globulin, or ovalbumin, this enzyme did hydrolyze casein, histone, and clupein. Schöffner & Truelle conclude, chiefly from these last findings, that the GE is not a peptidase. In the opinion of the reviewers, the assay methods employed in this paper largely invalidate the conclusions regarding the nature of the GE. The activity of the latter was measured by Van Slyke amino nitrogen, nephelometric, and viscosity methods. As was already pointed out, none of these procedures are able to distinguish between proteinases and peptidases. The data do seem to suggest that the HE was identical with Anson's cathepsin proteinase, while the inability of the GE to digest ovalbumin or serum proteins makes it seem likely that this enzyme was a peptidase.

The proteolytic enzyme systems of a number of snake venoms have been described by Ghosh, De & Chowdhury (62). Three different optimum pH values were reported for casein or gelatin substrates: one slightly alkaline, one distinctly acidic, and one near neutral pH. From the finding that the rates of inactivation of venom "trypsin" and pancreatic trypsin were approximately equal at elevated temperatures, it was concluded that the two enzymes are identical. The venoms hydrolyzed peptides containing glycine and leucine, and also attacked chloroacetyl tyrosine, this fact indicating the presence of carboxylpeptidase.

The mechanism and the quantum efficiency of the inactivation of crystalline trypsin has been studied by Verbrugge (63), with benzoyl-argininamide, casein and hemoglobin as substrates. The quantum yields increased with decreasing wave length. The inactivation of trypsin followed a simple exponential curve. The interpretation placed upon this fact was that a "one-hit" reaction is involved, which shows

that the active groups in the enzyme are independent of each other in their action.

A study of the pancreatic proteinase of the shark, *Cancharinus obscurus*, is reported by Boos (64). The proteinase was extracted by a modification of Anson's carboxypeptidase method. The optimum pH on hemoglobin was 6.5, somewhat more acidic than that of mammalian trypsin or chymotrypsin. The specific activity of the amorphous enzyme by the hemoglobin method, 0.0071 proteinase units per mg. protein nitrogen, is considerably less than the activities of crystalline trypsin or chymotrypsin. Also the shark pancreatic tissue was found to be only a sixth as rich in proteinase as an equal weight of beef pancreas.

Once again it has been demonstrated that peptide anhydrides (*l*-leucyl-*l*-glutamic acid anhydride) are not hydrolyzable by proteinase, e.g., pancreatin, pancreatic proteinase, or papain (65).

The liberation of histamine from living tissues by the action of trypsin and of papain but not by chymotrypsin has been demonstrated by Roche e Silva and co-workers (66, 67, 68). By means of experiments with inhibitors it was determined that the histamine liberating activity of papain ran parallel to its hydrolytic activity on the arginine-amide linkage. The conclusion was drawn that histamine is combined through an amide type of linkage with either arginine or lysine in cellular proteins.

Dragstedt (69) suggests that the ability of trypsin and other proteinases to digest mammalian tissues is due to the liberation of histamine which embarrasses the vitality of tissues dependent upon a vascular supply. On the other hand, cells not dependent upon a vascular supply, living bacteria, yeast, etc., are not injured by histamine, and thus are resistant to digestion by proteolytic enzymes.

#### PROTEOLYTIC ENZYMES OF HIGHER PLANTS

*Crystalline asclepain*.—The proteolytic enzyme of milkweed, the general properties of which were described in 1940, has been crystallized by Carpenter & Lovelace (70). The juice pressed from the roots of *Asclepias syriana* was the starting material, and the isolation procedure involved salting out the enzyme with at least half saturated ammonium sulfate at 5°. The yield was 6 to 8 gm. of yellow crystals from 3 kilos of roots. The purified enzyme was activated by cysteine, reduced glutathione, hydrogen sulfide, and cyanide. From careful electrophoretic measurements, the isoelectric point of crystalline as-

clepain was found to be at pH 3.11, in good agreement with the estimate of pH 3.2 for the isoelectric point of the partially purified proteinase of *Asclepias speciosa* (71). Carpenter & Lovelace also calculated the electric charge,  $\xi$ , of the asclepain molecule at different pH values, with the aid of the Helmholtz-Lamb equation.

*New proteinases.*—The continued interest of Latin-American investigators in proteolytic enzymes is indicated in two recent papers describing new proteinases. Jaffe (72) has obtained a papain-like enzyme from the juice of the shrub, *Tabernamontana grandiflora*, which he has named "tabernamontanain." This enzyme, prepared in a partially purified form by acetone precipitation, was several times as active as crude papain, as measured by milk clotting, or by the formol titration with gelatin or peptone. An interesting observation is that the juice collected in April was activated by cysteine or cyanide, while that obtained in July was not further activated by these reagents. The conclusion drawn was that no natural activator was present in the former case.

The proteolytic activity of the latex of *Euphorbia cerifera* has been studied by Castañeda, Balcazar & Gavarrón (73). The name "euphorbain" was proposed for this proteinase which was similarly activated by cysteine and cyanide, and inactivated by hydrogen peroxide. Another proteinase, which has also been named "euphorbain," has been obtained from the latex of the weed, *Euphorbia lathyris*, by the Australian workers, Ellis & Lennox (74). The enzyme, extracted from acetone-precipitated latex, was about as active as commercial trypsin or papain on gelatin, and had strong milk-clotting power. It is not yet known whether or not the two euphorbains have identical properties.

*Proteases of soybean.*—The proteolytic enzyme system of soybean seed is discussed by Laufer, Tauber & Davis (75). Aqueous glycerol extracts yielded the most active preparations. The optimum pH was 6.5 to 7 on gelatin and casein. As is generally the case with seeds, there was a marked increase in protease concentration upon germination of the soybean seeds.

According to Ham & Sandstedt (76), soybean also contains a proteolytic inhibitor, which like the trypsin inhibitor of pancreas, is probably a polypeptide. The soybean inhibitor was extracted from unheated meal at pH 4.2, at which pH the bulk of the soy proteins is insoluble. The extract was further deproteinized by treatment with kaolin. The inhibitor was tested by its retarding action on trypsin *in*

*vitro*. It was destroyed by autoclaving, and may be identical with a factor in soy beans which retards growth in chicks.

*Papain*.—The presence of natural activators, such as cysteine and glutathione, in plants containing papain-like<sup>4</sup> enzymes has been fairly well established. Gottschall (77) has observed that nearly-inactive papain can become activated spontaneously during the digestion of such varied materials as beef muscle, liver, and new beer. The activation apparently was due to the liberation of previously combined sulfhydryl groups in the substrates, and the addition of an activator was unnecessary. Crystalline chymopapain (free of natural activator) behaved in a similar fashion, in that it was rapidly activated by peptic digests of meat. The activating properties of the peptic digests were destroyed by treatment of the latter with sufficient iodoacetamide to abolish the nitroprusside test.

Lineweaver & Schwimmer (78) determined that crystalline papain is most stable in the pH range of 5 to 7 and is rapidly destroyed at 30° below pH 2.5 and above pH 12. It is stable for a period of 24 hours at 30° in 9 *M* urea. The nitroprusside test for sulfhydryl groups is negative even in the presence of cyanide, but becomes positive when the enzyme is denatured. The optimum reaction for proteolysis is at about pH 7 for denatured casein, egg albumin, and hemoglobin.

A paper by Elion (79), which is concerned with the relation between the improvement of wheat flour and the inhibition of papain-like proteases in flour, has provided a correlation between the chemical structures of a group of organic compounds (which are flour improvers) and their inhibitory action on papain. Whereas most of the inhibitors used have been inorganic oxidants, such as bromates, iodates, and perborates, Elion found that a group of organic oxidizing substances, all containing an enediol group with an adjacent carbonyl: ( $\cdot\text{C}(\text{OH}) : \text{C}(\text{OH}) \cdot \text{CO} \cdot$ ), were strong inhibitors of papain. Examples are dihydroxy maleic acid, reductic acid, reductone, and tetrahydroxyquinone. The presence of an enediol group alone did not impart inhibitory power to an organic compound.

*Comparison of proteinases*.—The available information on eleven plant enzymes which have been obtained in a comparatively purified or active form is summarized in Table I. The "ain" ending has been used in the naming of nearly all of the enzymes, in agreement with a recent proposal to this effect (80). The enzymes were obtained from a

<sup>4</sup> This adjective seems preferable to the alternative term "papainase."

variety of botanical genera. In most cases the latex of the plant was the source material. While the latex of a number of plants is quite rich in enzymes, and comparable in this sense to pancreatic or gastric juice (81), it is noteworthy that many other lactiferous plants are relatively lacking in such enzymes. Furthermore, there is no marked distinction between most latex-borne proteinases, such as papain, and intracellular tissue enzymes, such as bromelin. Four plant proteinases have now been obtained in crystalline form, but there is as yet very little information relative to the physical constants and amino acid constitution of the enzyme molecules. The wide difference in the isoelectric points of asclepain and papain is suggestive of differences in amino acid composition. The specificities toward synthetic substrates have been tested only in the cases of papain, bromelin, and ficin. While Table I appears to imply that bromelin acts on somewhat different substrates than papain and ficin, this is probably not so, for preliminary experiments suggest that the enzymes have similar specificities (52).

Antihelminthic properties *in vivo* and *in vitro* have been reported for certain of the proteinases listed in Table I, but there seems to be considerable difference of opinion at present regarding the therapeutic value of plant proteinases as antihelminthics and digestive aids. Some of the plant proteinases are unstable in acid solution and are destroyed by the gastric contents.

An interesting point which is not indicated in Table I is that all of the proteinases listed, except the last two, are inactivated by mild oxidation and reactivated by sulfhydryl compounds or cyanide. The last two enzymes, solanain and hurain, are quite unaffected by these inhibitors and activators, but are inactivated by iodine and nitrous acid. In addition, solanain and hurain are most active at alkaline, rather than neutral pH, and do not have strong milk-clotting power. Accordingly, hurain and solanain probably belong to a separate class of plant proteinases.

*Theory of activation.*—At the present time a large number of enzymes, including both proteolytic and certain nonproteolytic types, are known to have the property of being inactivated by mild oxidants and then largely reactivated by sulfhydryl compounds or cyanide. The majority of enzyme chemists have concluded that activators such as cysteine function as reducing agents. However, Bergmann and co-workers (3, 52) have pointed out that most oxidation-reduction studies of this type are complicated by the presence in the enzyme mate-

TABLE I  
CHARACTERISTICS OF PLANT PROTEINASES

Name Given to Enzyme	Botanical Origin			Crystalline Form	Isoelectric pH	Stability in Acid or Alkali	Synthetic Substrates	pH of Optimum Protein Digestion*	Milk Clotting Power	Anti-helminthic Action	Key References
	Common Name of Plant	Genus and Species	Source of Material								
Papain	Papaya	<i>Carica papaya</i>	Latex of green fruit	Needles, hexagonal plates	9	Unstable below pH 2.5 and above 12.0	Benzoyl-L-arginineamide, hippuryl amide, etc.	7-7.5	Strong	Digests Ascaris	52, 80 to 83
Chymopapain	Papaya	<i>Carica papaya</i>	Latex of green fruit	Needles, plates		Stable at pH 2		7	Strong		82, 85
Ficin	Fig	<i>Ficus carica, glabrata, doliaria</i>	Latex	Hexagonal plates	5 †	Stable at pH 2	Benzoyl-L-arginineamide, hippuryl amide	7	Strong	Digests Trichuris, Ascaris	52, 86, 87
Bromelin	Pineapple	<i>Anana sativa</i>	Fruit, leaves			Stable at pH 2-3	Carbobenzoxy-glycyl-L-glutamylglycine-amide	6-7	Strong	<i>In vitro</i> but not <i>in vivo</i>	52, 82, 83, 84, 88
Pinguinain	Maya	<i>Bromelia pinguin</i>	Fruit					3 (milk)	Strong	Digests Macracanthorhynchus	89
Asclepain	Milkweed	<i>Asclepias speciosa, mexicana, syriana</i>	Latex, roots	Rectangular plates	3.1	Unstable in acid or alkali		7-7.5	Strong		70, 71, 80
Mexicanain	Cuaguayote	<i>Pileus mexicanus</i>	Leaves, fruit						Strong		90
Tabernamontanain		<i>Tabernamontana grandiflora</i>	Sap, fruit					5-6 (gelatin)	Strong	Digests Trichuris, Ancylostoma	72
Euphorbain	Caper Spurget	<i>Euphorbia cerifera, lathyris</i>	Latex					6	Strong		73, 74
Solanain	Horsenettle	<i>Solanum elaeagnifolium</i>	Fruit			Stable in dil. alkali		8.5	Weak		80
Hurin	Jabillo	<i>Hura crepitans</i>	Sap		4-5 †	Stable in dil. alkali		8 (gelatin)	Weak	No action on Ascaris	91

\* On proteins precipitable by trichloroacetic acid, such as casein, and denatured hemoglobin and ovalbumin.  
† Region of minimum solubility.



rial of natural activators, which may undergo the reactions attributed to the enzymes. Bergmann and co-workers observed that the activation of papain-like enzymes by hydrogen sulfide or cyanide was reversed when these gases were pumped off *in vacuo*. They also found that dialysis of the enzymes to remove natural activators, or the precipitation of papain from cyanide solution by alcohol, resulted in a similar loss of activity. Bergmann (3) concluded that activators such as cyanide and sulfhydryl compounds can function, not only as reducing agents, but as coenzymes, and that the activation of papain-like enzymes involves the formation of (dissociable) enzyme-activator compounds.

Recently Winnick, Cone & Greenberg (87), in a paper dealing with the activation of crystalline ficin, have concluded that the apparent coenzymic function of cyanide and thiol compounds actually consists in a protection of the enzyme against oxidation by atmospheric oxygen or combination with heavy metals. A solution of ficin was found to retain its activity when freed completely of added cysteine by means of an efficient anaerobic dialysis procedure, or when added hydrogen sulfide or cyanide were removed, by evacuation in an atmosphere of purified nitrogen gas. When the dialysis was conducted in the presence of air, the enzyme was (reversibly) inactivated. The data supported the view that ficin and other papain-like enzymes are active in a reduced form and are inactivated by mild oxidation.

While a number of investigators have presented evidence that thiol groups, actual or potential, are necessary for the activity of such enzymes as papain and urease, additional critical chemical studies of sulfhydryl, phenolic, and possibly other groups, are needed to definitely establish the nature of the active group or groups in these enzymes.

An unusual hypothesis advanced by Scott & Sandstrom (92), is that the activation of papain involves a surface phenomenon. The finding that a series of homologous mercaptans activated papain in the same order as a similar series of heteropolar compounds lowers the surface tension of water, suggested that the activator exerted its effect at the enzyme-substrate interface. At relatively high concentrations the efficiencies of the activators were somewhat lessened, due presumably to their tendency to block the approach of substrate molecules. It may be in order to mention here the somewhat similar speculation of Crippa & Maffei (93) concerning the inhibitory effect which cyanide and reduced glutathione appeared to exert on peptic digests. The inhibitions,

which were in proportion to the concentrations of these reagents, were explained as being due to a blocking of the pepsin from the potential points of attack in the protein substrate.

### PEPTIDASES (EXOPEPTIDASES)

*General.*—Recent progress in the field of the peptidases has not been rapid. Johnson & Berger (8) have pointed out the lack of knowledge of the number and nature of the enzymes in most natural peptidase systems and the unsatisfactory chemical criteria which are often used as a basis for the differentiation of specific peptidases. A large number of papers, principally in German journals, describe results obtained with crude aqueous or glycerol extracts of tissues, without any serious attempts to isolate and study the component enzymes.

Outstanding by contrast is the work of Maschmann (94 to 100) on the fractionation of the peptidases in glycerol extracts of kidney, liver, cancerous tissue, etc., by means of fractional precipitation with acetone or ammonium sulfate. This investigator observed that peptidases of tissues frozen with solid carbon dioxide and dried *in vacuo* are easily extracted with water. Desiccation with acetone, on the other hand, caused substantial losses in activity. The enzyme activity most sensitive to the acetone treatment was that associated with the hydrolysis of alanylglycylglycine and glycylglycylglycine. The ability to split leucylglycine or leucylglycylglycine is not impaired by the acetone treatment, but there is a rapid loss of this activity in frozen, or frozen dehydrated preparations (96). Glycerol extracts were always more active enzymatically than aqueous extracts, but the two types of extracts differed markedly in their action on peptides containing glycine, alanine, and leucine. Based on an examination of the efficiency of the common extraction methods for peptidases, Maschmann (98) postulates the following types of peptidases: "exo" enzymes, of secretory origin; "lyo," or intracellular soluble enzymes; "desmo" enzymes, bound to protoplasm and liberated by proteolytic action; and "endo" enzymes, imbedded in the cellular structure, and insoluble. Upon examining liver, kidney, and tumor cells, Maschmann found that the soluble "lyo" enzymes predominated in each case.

A highly active leucylpeptidase has been prepared from beef muscle by Schwimmer (101). The purified preparation hydrolyzed leucylglycine and leucyldiglycine, but not simple glycine or alaninepeptides. The enzyme resembled the leucylpeptidases found in hog erepsin and

in various plants and bacteria by Berger & Johnson, in its optimum pH of 8.3, and in its activation by magnesium and manganese salts. The original beef muscle appeared to contain other peptidases which were eliminated by the purification procedure.

Using glycylglycyl-*D*-alanine as substrate, Levy & Palmer (102) found that there is an accumulation of an aminopeptidase during the development of the chick embryo. Polypeptidase and dipeptidase activities have been found in egg white (103). The enzymic activity is greatly increased if the fresh egg white is maintained at pH 8 for four days. Maschmann (99) found that the dipeptidase activity of yeast is greatly increased by manganous and cobaltous ions; particularly the latter which raises the activity by as much as 2000-fold. Enormous increases in peptidase activity by divalent metal ions are produced also in blood serum.

A recent paper by Smith & Bergmann (104) is a substantial contribution to the problem of the differentiation and purification of the various peptidases present in swine intestinal mucosa. The following enzymes were studied: (a) An "*L*-leucyl-aminoexopeptidase" which corresponds to Linderström-Lang's leucylpeptidase. This enzyme was separated from most of the other peptidases in an aqueous extract of mucosa by precipitation with acetone, followed by fractionation with ammonium sulfate. (b) A peptidase which, unlike the preceding enzyme, is not activated by manganese ions. This enzyme, tentatively classified as a "glycine-imidoendopeptidase," hydrolyzed *L*-leucyldiglycine, but not *L*-leucylglycine. (c) Prolidase, which was activated by manganous ions, but not by divalent cobalt, zinc, magnesium, or copper ions. This enzyme hydrolyzed glycyl-*L*-proline and glycyl-*L*-hydroxyproline. (d) One or more endopeptidases which hydrolyzed carbobenzoxyglycyl-*L*-prolineamide and carbobenzoxyglycyl-*L*-hydroxyprolineamide. (e) Enzymes not yet well characterized, which hydrolyzed a variety of peptides. These investigators have come to doubt the existence of enzymes which are exclusively dipeptidases.

Neubeck & Smythe (105) have studied an enzyme in guinea pig liver which hydrolyzed glutathione, with the liberation of cysteine. Whereas the intact liver maintained a considerable concentration of glutathione with very little free cysteine, it was found that the hydrolysis of glutathione by enzyme preparations *in vitro* approached completion. The optimum pH of cysteine production was 8.4 to 8.7 for liver homogenate, and 9.0 for a partially purified enzyme prepara-

tion. Dialysis of the enzyme resulted in its inactivation. While the nature of the dialyzable component was not known, it appeared to be a very stable substance. One might suggest here that the dialyzable component was a metallic ion, and it may be of interest to test the enzyme on other peptides.

Ägren (106) has shown that liver incubated with highly purified intestinal amino peptidase was effective in pernicious anemia treatment. He concludes that this enzyme is the extrinsic anti-anemic factor of Castle.

*Metal ion activation.*—It has been known for some time that a number of the peptidases are activated by certain divalent metal ions, manganous ion usually being the most potent (8). Recently it has been shown that the activation of aminopeptidase by manganous ion, like the activation of arginase, is a time reaction (104). Maschmann (95) has observed that the rate of hydrolysis of different peptides by the same enzyme extract may be maximally activated by different metal ions. Thus with extracts of kidney, liver, and intestine of rabbit and guinea pig, with chick embryo, and with mouse sarcoma extracts, it was usually found that manganese was the best activator for leucylglycine and leucylglycylglycine; magnesium for alanyl glycine; cobalt for glycylglycine, diglycylglycine, and alanyl glycylglycine; zinc for glycylleucine and glycylalanine. To obtain hydrolysis of *d*-peptides may require activation by manganous ion and a sulfhydryl compound, i. e., cysteine (97).

*d-Peptidases.*—Investigation of the enzymic hydrolysis of peptides composed of *d*-amino acids was stimulated by the report of Kögl & Erxleben (107) concerning the presence of *d*-amino acids in tumor proteins, and their hypothesis that normal tissues were unable to cope with cancerous growths because they lacked enzymes capable of attacking *d*-peptide bonds. While the validity of Kögl's claims have become ever more doubtful, the ability to hydrolyze *d*-peptides has been shown to be widespread in biological materials. Extracts of many animal organs and tissues (94 to 100, 108 to 117), of blood sera (97, 99, 118), neoplastic material (97, 110, 113 to 117), germinating seedlings and the growing parts of higher plants (110), and microorganisms can hydrolyze *d*-peptides in such racemates as glycyl-*dl*-alanine, *dl*-leucylglycine and *dl*-leucylglycylglycine, and leucyl-*dl*-leucine. The extracts generally act very much better on the *l*-components of the peptides. In animal tissues this activity is strongest in kidney, less so in liver (95), and can be demonstrated in normal blood serum only after

activation by manganous ion and cysteine (99). *d*-Peptidase activity is found in tumors, but the parenchymal cells of fresh normal tissues were found to be a richer source of enzyme activity than embryo or mouse carcinoma cells.

Untreated glycerol extracts of fresh tissues or aqueous extracts of dry tissues do not split optically pure *d*-leucylglycine (96). Hydrolysis of this peptide occurs only after activation by manganous ion and cysteine, scarcely so by manganous ion alone under aerobic conditions (97).

There is little reason to infer that there is any correlation between *d*-peptidase activity and the growth of neoplasms. Bamann & Schimke (110) sum up the evidence for this conclusion as follows: (a) *d*-peptidase activity is found in most animal organs and tissues; (b) the *d*-peptidase activity of carcinoma and metastasized tissue is not greatly different from that of normal tissues; (c) there is no distinction between the ratio of the activities on *l*- and *d*-peptides of extracts from normal and from cancerous tissues; and (d) the activation and inhibition behavior of the peptidases of normal and cancerous tissue is the same.

There is considerable support for the hypothesis that the hydrolysis of *d*-peptides is caused by a distinct enzyme or group of enzymes. The evidence for this is that *d*-peptidase activity is more labile than *l*-peptidase activity and is destroyed by dialysis (96, 109, 110), aseptic autolysis (111, 112), unfavorable pH and temperature (110), and acetone precipitation (96, 104), while *l*-peptidase activity persists. By partial purification it has been possible to increase the degree of *d*-peptidase action to a point where the ratio of *d* to *l* enzyme activities was about equal (96). Maschmann (96, 97) considers that a number of enzymes are involved, since the most highly purified preparations capable of splitting either *l*- or *d*-leucylglycine showed little or no action on other available di- and tripeptides. Bayerle & Borger (112), on the contrary, consider that the same enzyme is involved in the hydrolysis of *l*- and *d*-peptides. They suggest that it is the same apoenzyme with two different coenzymes. In their experiments, they observed that there is a parallel increase or diminution of *l*- and *d*-activity by acetone precipitation. Taking into proper account the necessary activating action of manganese or manganese and cysteine on peptidases, they were unable to effect a separation of the capacity to hydrolyze both *d*- and *l*-peptides. The evidence for separate enzymes, however, is the more convincing.

The unfavorable rate, or even complete lack of hydrolysis of the *d*-component of *dl*-leucylglycine, has been explained as being due to an intense inhibition by the liberated *l*-leucine (110). In the case of leucyl-*dl*-leucine, Abderhalden & Aberhalden (109) observed that *d*-leucine inhibited the hydrolysis of the *l*-peptide, and hydrolysis of the *d*-peptide was inhibited by *l*-leucine, but the latter was even more strongly inhibited by the *d*-leucine. In contrast, Maschmann (97) observed that *l*-leucine apparently does not inhibit the hydrolysis of the *d*-component of glyceryl-*dl*-leucine.

## APPLICATIONS

Considerable information relative to the preparation and uses of proteolytic enzymes in industrial and medicinal chemistry is presented in the book, *Enzyme Technology* by Tauber (119). The increasing practical importance and varied commercial applications of proteolytic enzymes are well illustrated in this volume.

It may be of interest to mention also some additional applications of proteases to medical and theoretical problems. Plentl & Page (23) have studied the action of four different crystalline proteases on angiotonin. All of the proteases destroyed the angiotonin *in vitro*. By regarding the enzymes as analytical reagents, an attempt was made to interpret the experimental findings on the basis of Bergmann's specificity studies by comparing the pH optima and proteolytic coefficients of the reactions with values determined for synthetic peptides of known structure. It was concluded that angiotonin contained a free terminal amino group and a free terminal carboxyl group, and that the simplest formula satisfying the specificity requirements of the enzymes was tyrosylarginylglutamylphenylalanine, or a combination of amino acids with similar properties. The course of the angiotonin hydrolysis could not be followed by measuring the increase in amino nitrogen or free amino acids, and a bioassay method was used which measured the destruction of the pressor substances. The inapplicability of the chemical methods here seems curious, in view of the apparently ample angiotonin concentrations and the rapid action of the enzymes. It seems also noteworthy that crystalline angiotonin fails to give a positive biuret test (120).

In a study dealing with the chemical composition of *Rickettsia prowazekii*, the etiological agent of epidemic typhus, Cohen & Chargaff (121) described the use of trypsin and chymotrypsin for the dis-

integration of the rickettsial organism. This technique resulted in a larger yield of antigenic material. The material liberated included two antigens, which were apparently protein-carbohydrate complexes, resistant to proteolysis.

While acid hydrolysis of proteins is generally employed in the preparation of amino acid mixtures for nutritional purposes, reference may be made to several instances in which enzymic digests of protein were utilized. Madden *et al.* (122), in comparing the effectiveness of casein digests for long continued plasma protein production in dogs, concluded that "amigen," a digest of casein by pork pancreas (Mead Johnson), provided good nitrogen retention and fairly good plasma protein production. The tolerance, however, was much less upon intravenous injection than for certain mixtures of pure amino acids. An acid hydrolysate of casein fortified with tryptophane (Frederick Stearns) afforded bare nitrogen equilibrium, and produced virtually no plasma protein.

A standardized, sterile papain hydrolysate of meat, freed completely of undigested proteins and allergens, and put up in saline with added glucose and vitamins, has been successfully used in the treatment of severe starvation in India (123, 124, 125). This mixture given intravenously was more effective than blood serum in reducing the death rate from inanition. The hydrolysate also was beneficial in the treatment of hemorrhagic shock in cats.

The authors preferred digesting the meat with papain at an elevated temperature (50°), rather than with trypsin, because in their estimation, the higher temperature reduced bacterial activity and the chance of liberating histamine.

Magnusson (126) has reported very favorable results in the use of an enzymic digest of protein as a food for premature infants. The amino acid mixture, known as "aminosol" (Vitrum, Stockholm), was prepared by the digestion of casein by proteases, followed by dialysis. The aminosol is said to contain 80 to 85 per cent free amino acids and is used with glucose and a salt mixture. The solution, given first by catheter, and later orally, was well tolerated, and gave greater gains in weight than equal amounts of unhydrolyzed casein. According to the author, the amino acids seemed to be utilized almost quantitatively, as judged from the gains in weight.

That the hydrolytic action of a mixture of proteases on a protein can be as effective as that of mineral acids was suggested by the report of Hotchkiss (127) that pancreatic extract, followed by an ex-

tract of intestinal mucosa on crystalline lactoglobulin caused about 97 per cent of the cleavage produced by twenty-four hour hydrolysis with 6 *M* hydrochloric acid. This observation also suggests the possibility (as yet untried) of employing enzymatic hydrolysis in connection with the determination of the amino acid content of proteins.

Bayerle & Borger (128), in an attempt to isolate *d*-glutamic acid from tumor tissue, have employed peptic digestion, to avoid the racemization presumably encountered by Kögl & Erxleben with acid hydrolysis. After treating protein material from carcinoma tissue repeatedly with commercial pepsin, Bayerle & Borger were unable to isolate *d*-glutamic acid from the enzymic digest. The authors acknowledge that other proteinases and peptidases should be tried, since there is no evidence that pepsin liberates free glutamic acid from proteins.

A study by Tiselius & Grönwall (129) of the action of crystalline proteinases on purified tuberculin has indicated that the nonprotein cleavage products of the latter protein can retain a considerable degree of biological activity. In their study, Tiselius & Grönwall employed a highly active tuberculin of molecular weight 10,500, prepared by Seibert's method. They found that pepsin at pH 2 or 3.5 split roughly 11 to 12 peptide bonds per tuberculin molecule, as measured by titration of liberated amino groups, and that there was a resultant loss of almost 90 per cent of the biological activity, as measured by skin reactions on animals previously inoculated with virulent cultures of tubercle bacilli. However, the activity which remained was considerable, while the digestion of protein (to polypeptides) was fully complete (as judged from precipitation tests with trichloroacetic acid and ammonium sulfate). When digestions were made with trypsin or chymotrypsin, the hydrolysis of the tuberculin was far more extensive, and the products, which were chiefly dipeptides, had no biological activity.

## AMIDASES

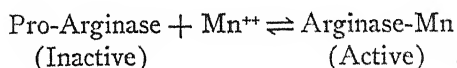
Attention among this group of enzymes is still mainly devoted to the study of urease and arginase. Renewed interest, however, has been aroused in glutaminase because of the recent observations that glutamine is readily synthesized by a number of tissues and that this compound probably is the source of the ammonia excreted in the urine (130).



## ARGINASE

*Activation.*—One of the intriguing problems concerning arginase is the mechanism of its activation by the divalent cations of manganese, cobalt, and nickel. This problem has general interest, since a large number of enzymes of varied character are also activated by these cations.

Recent investigations have disclosed that the activation of arginase is a reversible process; being governed by time, pH, temperature, and the type and concentration of the activating ion (131, 132). Each arginase preparation has a potential maximum activity, which once attained, cannot be exceeded, but may be lowered by changing the experimental conditions. The activation of arginase seems best explained by the following scheme, using manganous ion for illustration:



The complete activation of arginase in the presence of minimal amounts of activating cation may require a number of days. The time of activation can be greatly decreased by increasing the temperature or activator concentration. In equal concentrations, cobalt has several times the accelerating effect of manganese, but the final activation produced by cobalt is no greater and may be less than that produced by manganese. The role of manganese and cobalt might be assumed to be purely catalytic were it not for the fact that inactivation of arginase occurs very rapidly if the concentrations of manganous or cobaltous ions are lowered. This can be accomplished by dialysis, or by the addition of citrate or borate buffers, which probably form complexes with these cations. When these buffers are added, a proportionate reduction in arginase activity occurs as rapidly as the measurements of activity can be carried out. On the basis of this evidence, it seems probable that the active arginase is an easily dissociable compound of protein and activating cation.

*Purification.*—Hunter & Downs (131) have produced a three to four fold increase in the units of arginase per mg. of nitrogen by fractional precipitation of liver extract with acetone and re-solution of the precipitate in 75 per cent aqueous glycerol. Their aim was to have an active and stable preparation suitable for use for the quantitative determination of arginine. Mohamed & Greenberg (132) prepared arginase of high potency by extraction of ground liver with 5 per

cent sodium acetate and then by fractional precipitation, first with lead acetate and then with acetone. The isolation procedure is based on the preferential denaturation and precipitation of nonarginase proteins by lead acetate and acetone. This method yielded an increase of over twenty fold in enzyme activity per mg. of nitrogen as compared with that of the initial sodium acetate extract. The best previous preparations (133) appear to have had about one half to one third the activity of the above.

*Properties.*—Arginase prepared as above still contains three to four protein components when examined electrophoretically. The isoelectric points of the two main protein components are both at approximately pH 5.0 (132, 134).

Arginase is quite a stable enzyme (131, 132). At refrigerator temperature, a beef liver preparation in solution lost activity at the rate of about only 1 per cent a month. Arginase is stable to many heavy metals, being inactivated only by silver and mercuric ions among those tried. It is not readily destroyed by oxidizing agents, e.g., hydrogen peroxide, but it loses its activity upon addition of iodine; perhaps as the result of iodination of aromatic radicals in the enzyme molecule.

*Kinetics.*—The kinetics of the arginine-arginase reaction have a number of interesting aspects (135). The influence of substrate concentration on the rate of arginine hydrolysis lends itself readily to interpretation by the Michaelis-Menten equation, on the basis of an active intermediate composed of one molecule each of enzyme and substrate. The magnitude of the enzyme-substrate dissociation constant was observed to vary with pH, the graph of dissociation constant against pH yielding a U-shaped curve with a minimum at about pH 8.0. On the assumption that the active enzyme intermediate is composed of the monovalent cation of arginine and the anionic form of arginase, the following equation was developed for the true enzyme-substrate dissociation constant (135).

$$Ks \text{ (true)} \left( \frac{K_1}{C_H} + 1 \right) \left( \frac{C_H}{K_2} + 1 \right) = Ks \text{ (experimental)}$$

In this equation  $Ks \text{ (experimental)}$  represents the enzyme-substrate dissociation constant derived from the experimental data at different pH values,  $K_1$  is the dissociation constant of the arginine ( $9 \times 10^{-10}$ ) (136), and  $K_2$  is the dissociation constant of the enzyme (approximately  $1 \times 10^{-7}$ ). By means of the above equation,  $Ks \text{ (true)}$  was calculated to be  $4.25 \times 10^{-3} M$ .

The temperature of optimum activity for liver arginase lies between 45 and 50°. Above 50° the rate of inactivation is rapid. The rate of thermal destruction of the enzyme follows a first order reaction. At 50° the half life of the arginase is 130 minutes. The critical thermal increment of inactivation is about 35,000 calories.

*Biological distribution.*—Dounce (137) has demonstrated that in the liver, arginase occurs in the cell nuclei in high concentration. Greenstein *et al.* (138, 139) observed that the arginase activity of rat and mouse hepatomas was lower than that of corresponding normal liver tissue. Regenerating liver tissue had the same activity as the normal tissue.

Arginase is found in the mucosa of the small intestine of various animals (140). At its pH optimum, it is stated to act on *d*(—) arginine. Like liver arginase, the intestinal arginase is activated by manganous ion and inhibited by *l*(—) ornithine.

The hemolymph of the snail *Helix pomatia* is reported to contain arginase which is not activated by manganous ion (141).

Srb & Horowitz (142) have demonstrated by means of a study of the gene mutations of the bread mold (*Neurospora crassa*) that an ornithine-arginine cycle occurs in this organism similar to that proposed by Krebs & Henseleit (143) for the liver. The evidence for this is that upon irradiation of the mold three types of *arginineless* mutants have been isolated; namely, one able to grow on arginine, ornithine, or citrulline; another able to grow on arginine or citrulline but not ornithine; and a third specifically requiring arginine. Part of the evidence for the cycle is the demonstration of the occurrence of arginase in *Neurospora*. The ornithine-arginine cycle is coupled with the presence of urease which hydrolyzes the urea formed to ammonia and carbon dioxide. The mold tissues can make use of the ammonia but not of urea directly. These observations throw a new light on the reported presence of arginase in the legumes, e.g., jack beans and soy beans.

Certain objections to the validity of the ornithine-arginine cycle in the production of urea by the mammalian liver have been answered by the experimental work of Krebs (144) and of Gornall & Hunter (145). Direct support of the cycle is offered by the demonstration of the latter authors that citrulline exerts the same kind of catalytic effect as ornithine upon urea formation, and that the production of urea under the catalytic influence of ornithine is accompanied by an accumulation of citrulline. The proposal of Bach (146) that glutamine is

the principle ammonia carrier in place of ornithine and citrulline has been countered by Krebs with the observation that urea formation was equally rapid in the presence of ammonium glutamate or ammonium lactate as of glutamine. Krebs suggests that glutamine is first hydrolyzed to ammonium glutamate by glutaminase and the ammonium ions then enter in the ornithine-arginine cycle. Other alternative mechanisms that have been proposed for urea synthesis have been shown to be rather improbable by Krebs (144). There still remains to be satisfactorily resolved the observations of Bach *et al.* (147) that urea synthesis readily takes place in liver slices from ammonium lactate when the arginase is presumably inhibited by ornithine and the fact that in liver slices arginase activity apparently is inhibited while urea synthesis is accelerated by oxygenation.

The control of nitrogen metabolism in mammals by the endocrine secretions is reflected in changes in the arginase activity of the liver. Fraenkel-Conrat, Simpson & Evans (148, 149) observed that hypophysectomy lowered the arginase activity to about half the normal level, and adrenalectomy even more. Administration of adrenocorticotrophic hormone reversed the decrease in liver arginase of the hypophysectomized rat and also increased the arginase activity of normal rats. Growth hormones of the pituitary, on the other hand, decreased the liver arginase activity of both hypophysectomized and normal rats. The action of the adrenocorticotrophic hormone is in harmony with its established stimulation of protein breakdown, and the effect of the growth hormone agrees with its known action of decreasing the formation and excretion of urea. The authors suggest that the decrease in arginase is caused secondarily in response to a lessened need for this enzyme in animals with a positive nitrogen balance.

An investigation to determine which of the adrenocortical hormones was responsible for the reversal of the decrease in liver arginase activity produced by adrenalectomy showed that corticosterone, 11-dehydrocorticosterone, and 11-dehydro-17-hydroxycorticosterone produced increases in arginase, while desoxycorticosterone and male and female sex hormones had no effect (148). Kochakian (150) has confirmed the lack of effect of the sex hormone steroids on the arginase activity of the liver or the intestine. However, he found there were very marked changes in kidney arginase. Many steroids increased the arginase content of this organ as much as 600 per cent per gm. of tissue, or 1300 per cent for the total organ. Increase in

arginase activity was always associated with an increase in kidney size. The most potent steroids were 17-methyltestosterone, testosterone, testosterone propionate, and 17-methylandrostanediol-3 ( $\alpha$ ), 17 ( $\alpha$ ). The presence of the 17- $\alpha$ -hydroxyl group appeared to be essential for the influence on kidney arginase. Castration was found to increase the arginase content per gm. of kidney but not the total enzyme content of the organ. Kochakian suggests that the changes in arginase activity are connected with the protein anabolism and not the nitrogen catabolism of the animal body.

A decrease in the arginase activity of the liver is produced by dietary manganese deficiency (151, 152). The work of Boyer and co-workers (151) indicated that there was an actual decrease in enzyme content but Shils & McCollum (152) concluded that the reduced activity was caused by insufficient manganese for the complete activation of the enzyme, since upon addition of manganese to the excised tissue, the activity is restored to normal. It is interesting that the restoration of activity by manganous ion was observed only when arginine carbonate was used as the substrate and not with arginine monohydrochloride (152).

#### UREASE

*Activation of sulfhydryl groups.*—The reversible inactivation of urease, like that of the papain-like enzymes, has been explained on the basis of the reversible oxidation-reduction, or substitution of the hydrogen of specific sulfhydryl groups in the molecule that are less reactive than the usual thiol group (153). Upon addition of one mole of mercuribenzoate, of iodoacetamide, or of one equivalent of iodosobenzoate per 21,300 gm. of urease there is no decrease in the catalytic activity of the enzyme. Addition of two moles or equivalents of the above reagents abolishes the enzymatic activity.

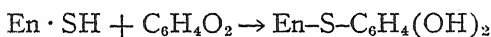
The interpretation placed on the above results is that urease contains two forms of sulfhydryl groups: one, which is immediately available to the action of nitroprusside, mercaptide-forming metallic compounds, iodoacetamide, and certain oxidizing agents but is not essential for the activity of the enzyme; and a second form, still capable of rather rapid interaction with mercuribenzoate and concentrated solutions of iodosobenzoate and iodoacetamide, but relatively less available to the action of certain other reagents (nitroprusside, ferricyanide). The inactivation is fully reversed by cysteine.

The two types of sulfhydryl groups described above are not the

only ones present in urease. Analysis of the completely denatured enzyme protein yields a value of approximately five thiol groups per 21,300 grams.

Numerous substances have been reported to inhibit or to inactivate urease. These include ascorbic acid (154, 155), quinones, *p*-phenylenediamine and substituted diamines (156), and the following compounds in decreasing order of effectiveness: bromo- and chloro-picric, isothiocyanate, chloracetophenone, dichlorodiethyl sulfone, dichlorodiethyl sulfide, and dichlorodiethyl sulfoxide (157). In nearly all instances the inhibition can be explained by the oxidizing properties of the inhibitor, or its ability to block the sulfhydryl groups in the urease molecule. Where the possibility of oxidation is not immediately apparent, as with ascorbic acid and hydroquinone, the inhibition may be traced to an oxidized impurity (quinone) in the compound in question (156).

The inhibiting action of ascorbic acid on urease activity is associated with the oxidation of the vitamin and is catalyzed by traces of copper (155). With many of the above compounds (ascorbic acid, quinones, phenylenediamines) inhibition may be prevented but cannot be reversed by the addition of cysteine. Where the process cannot be reversed by cysteine, Potter (156) argues that inhibition of sulfhydryl-containing enzymes takes place according to an irreversible reaction of the type



*Biological distribution.*—Urease is found in the red corpuscles of the rat, rabbit, and man (158). The activity is increased by autolysis and is inhibited by blood plasma. The pH optimum of red cell urease is 7.0. Urease also occurs in liver and spleen. There is a very high urease activity in the ingesta of the bovine rumen (159). The activity is sufficiently high to readily convert fed urea to ammonia within an hour. The urease of the rumen contents resembles jack or soy bean enzyme.

*Determination of urease activity.*—For the estimation of urease, Van Slyke & Archibald (160) recommend the use of 5 per cent egg albumin to protect dilute solutions of urease of high activity against inactivation. When the albumin alone is insufficient to maintain the enzyme stability, this can be accomplished by the addition of glutathione or sodium thioglycolate to the albumin-containing solution. Methods for the determination of urease activity by manometric,

titrimetric, and colorimetric procedures have been developed by these investigators. The analyses are performed under conditions in which the enzyme activity obeys the relationship

$$E = \frac{x}{Kt}$$

where  $E$  is the enzyme activity,  $x$  is the amount of carbon dioxide or ammonia that is formed,  $K$  is the velocity constant and  $t$  is the time. If the time is maintained constant,  $x$  is directly proportional to the urease concentration. From the amount of carbon dioxide formed (determined manometrically) or the ammonia formed (determined either titrimetrically or colorimetrically), the activity is evaluated in terms of Sumner & Graham (161) urease units. This unit is represented by the amount of urease capable of producing 1 mg. of ammonia nitrogen in five minutes at 20° in buffered phosphate solution.

For use in the estimation of urea in connection with amino acid determinations or the determination of arginase activity, Archibald (162) suggests the elimination of canavanine from urease by dialysis. Canavanine is slowly attacked by arginase, yielding urea and canaline. To eliminate canavanine and possibly other ammonia forming precursors in urease, Sumner & Sisler (163) propose that crystalline urease be employed for the estimation of urea instead of the crude extracts now used. These authors state that crystalline urease can be prepared easily from commercial jack bean meal.

#### GLUTAMINASE AND ASPARAGINASE

A specific method for the estimation of glutamine in blood and biological tissues, in normal and pathological conditions, based on the specific hydrolyzing action of glutaminase on this compound has been developed by Archibald (164). The glutaminase for this purpose is prepared from the cortex of either dog or beef kidney. The glutaminase of dog kidney was found to be more active and more specific than the enzyme from the kidneys of beef. Glutaminase is present in normal kidneys and there is a reduction in activity in the kidneys of nephritic subjects.

The kidney glutaminase appears to be mainly in a cell bound form. It has not been possible to increase the ratio of enzyme activity to protein content by any of the methods tried. The cyanide used in its preparation augments the glutaminase activity and depresses the liberation of ammonia from asparagine and from  $\alpha$ -amino groups. The

pH of optimum activity is at about 7.2 to 7.5. The enzyme is very unstable and extracts lose an appreciable amount of their activity daily either at 3° or in a frozen state. Intact, frozen dog kidneys, however, retain their glutaminase activity for over a year.

The glutaminase of the kidney appears to be quite specific. Phenacetyl glutamine and benzoylglutamine are not attacked. Small amounts of ammonia are liberated at a constant rate from *dl*-citrulline. Glutaminase from rat brain slowly hydrolyzes isoglutamine. Glutaminase, it was observed, was inhibited by bromosulfalein (disodium sulfonate of phenoltetrabromophthalein) and atabrine, the former being particularly potent.

The autolysates of certain bacteria, e.g., *B. pyocaneum* and *B. proteus vulgare* contain an asparaginase, which has its optimum activity at pH 8.0 (165). The asparaginase content of these organisms can be increased by the addition to the culture medium of alanine, glycine, *p*-aminobenzoic acid, and asparagine or aspartic acid.



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## NONPROTEOLYTIC, NONOXIDATIVE ENZYMES

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The reviewers have selected sixteen of the nonproteolytic, non-oxidative enzymes for consideration. The enzymes involved in transformations of phosphorus compounds that are of importance in carbohydrate and phosphorus metabolism receive attention in Chapter XI. Only brief mention of background will be made for those enzymes considered in detail in last year's *Annual Review of Biochemistry*. As is customary, the references cited are a selected rather than a comprehensive list. The *in vitro* properties of the enzymes are considered especially, rather than their physiological functions. It may be of interest, however, to list some of the hydrolytic enzymes that have received attention recently with respect to their physiological or pathological significance: cholinesterase has been studied in relation to the transmission of nervous impulse; carbonic anhydrase in acid-base equilibrium and in the nerve system; phosphorolytic enzymes in carbohydrate metabolism; mucinolytic enzymes (hyaluronidase, mucinase) in relation to the permeability of connective tissue, invasive power of bacteria, and fertilization; arginase in protein metabolism; a number of enzymes in relation to cancer; and procaine esterase in relation to toxic goiter.

Interest in the relation between enzymes and vitamins is reflected in studies of the vitamin content of purified proteins and enzymes. The amounts of biotin and *p*-aminobenzoic acid in catalase, rennin, urease, yeast polypeptidase, and phosphorylase were so small that they were considered to be only impurities (1). In highly purified pancreatic amylase, inositol was found in sufficient quantity (4.1 mg. per gm.) to be regarded as an integral part of the enzyme, even if the molecular weight were no more than 44,000 (2). In the other ten enzymic and eleven nonenzymic proteins studied only insignificant quantities of any of eight B vitamins were found, with the possible exception of carboxylase, which contained about 0.14 mg. of nicotinic acid per gram. Thus if the B vitamins, other than riboflavin, thiamine, and nicotinic acid, are enzyme constituents, then there exists a multi-

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plicity of enzymes that are not at present available in concentrated form (2).

Meyer (3) and Laurence (4) reported that biotin increased the lytic activity of lysozyme and avidin preparations and made the attractive suggestion that the lysozyme activity of avidin concentrates might be due to the avidin-biotin complex. However, Alderton *et al.* (5) found that biotin did not stimulate the lytic activity of purified crystalline lysozyme (6) and that lysozyme contained only a trace of biotin (0.009  $\mu\text{g. per gm.}$ ). Since about 85 per cent of the lytic activity of egg white was accounted for by the activity of an isolated crystalline protein it must be concluded that biotin is not a prosthetic group or a constituent of the principal lytic agent in egg white. Furthermore, lysis by avidin preparations, whether containing or not containing some lysozyme, was not increased by the addition of biotin, nor did avidin inhibit the lytic activity of lysozyme (5). These results, combined with the fact that the purified lysozyme was essentially free of avidin, argue against a relation between lysozyme and biotin or avidin. No obvious explanation of the discrepancies in the results by the different investigators is evident, although it seems logical to postulate a lytic principle other than the lysozyme isolated in high yield by Alderton *et al.* (6) or to postulate that the differences arose from differences in experimental method, since the assay and preparation methods used by Meyer and by Laurence were not fully described.

Current investigations of the mode of action of enzymes have employed almost exclusively the concept that substrates and inhibitors form complexes or compounds with the enzymes. Reversible compound formation has not always been necessarily assumed (7). For hydrolytic enzymes the existence of enzyme-and-substrate intermediate compounds has not been demonstrated by spectroscopic means, as in the case of peroxidase, nor by other direct means as opposed to deductions from kinetics. One reason for this is that water, because it is the solvent as well as a reactant, cannot be removed from the system to prevent decomposition of the intermediate compound.

The interpretation of kinetic data in terms of the combination of enzymes with their substrates and inhibitors has been limited generally to cases where the decrease in concentration of substrate or inhibitor due to combination with the enzyme could be neglected, i.e., where the molar concentration of enzyme is much less than the molar concentration of substrate or inhibitor. Straus & Goldstein (8, 9) have developed, in more detail than previous investigators (10), mathe-

mathematical and graphical treatments for cases in which the simplification mentioned above would not apply. They derived the rigorous equations for the systems of interest, which may be combinations of individual reactions ( $E + S \rightleftharpoons ES$ ,  $ES \rightarrow E + P$ ,  $E + I \rightleftharpoons EI$ ,  $E + nI \rightleftharpoons EI_n$ , etc., where  $E$ ,  $S$ ,  $P$ , and  $I$  represent enzyme, substrate, product, and inhibitor respectively). The equations were normalized by the expression of enzyme, substrate, and inhibitor concentrations in multiples or units of the appropriate dissociation constants ("specific concentrations"). In this way a single parameter expresses the kinetics for all enzyme systems of the same type. The derived second-order rigorous equation reduces, of course, to the more familiar kinetic equation when the concentration of inhibitor or substrate is not reduced significantly by combination with the enzyme, and it reduces to another simple form when the concentration of inhibitor (or substrate) is reduced nearly to zero by reversible combination with the enzyme (nearly all combined with the enzyme). In the latter case the inhibition is related to the total inhibitor concentration in a way that approximates the law of definite proportions, but the combination is in accord with the mass action law and is not necessarily irreversible. The concentration regions in which the limiting equations are applicable were designated zones A and C and the intermediate region as zone B. For the same type of system the "zones" always fall within the same "specific concentrations" but, of course, not within the same absolute concentrations; thus the usefulness of the term "specific concentration" is illustrated. These mathematical analyses may apply in pharmacological studies where high, localized enzyme concentrations are encountered and in cases where the dissociation constants are very small (e.g.,  $10^{-8}$  to  $10^{-6}$   $M$  physostigmine inhibits cholinesterase activity in serum). The cholinesterase (serum)-acetylcholine-physostigmine system was used to illustrate the application of the mathematical treatment (8, 9). Unfortunately the system used for illustration was found to fit (or nearly fit) the zone A equation. However, the analyses of competitive inhibition, noncompetitive inhibition, dilution effect, inhibitor destruction, etc., may be of assistance in drawing conclusions from kinetic data and in conducting kinetic studies.

#### AMIDASES

*Arginase*.—Hunter & Downs (11) refined the method of determining arginase. They showed that activation (122 per cent increase in activity) by cobalt is a slow reaction at 20° and 37°. At these tempera-



tures the activation was not complete for a matter of hours, whereas at 50° the activation was complete in twenty minutes or less. The time required for activation indicates that the reaction is not ionic. The activated enzyme is more stable than the unactivated enzyme.

*Glutaminase.*—An enzyme method based on the hydrolysis of the amide group by glutaminase is now available for the determination of glutamine (12, 13). The method is specific for glutamine in the absence of purine nucleosides bearing amino groups. Because of the relatively small amounts of these compounds in plasma the method may be used for the determination of glutamine in plasma. The enzyme (an emulsion of dog kidney), although fairly unstable, is readily prepared from frozen kidney, which retains its activity for more than a year. The presence of 0.0025 *M* potassium cyanide in the assay mixture reduces the action of the kidney emulsion on  $\alpha$ -amino groups to insignificant levels, reduces the slow liberation of ammonia from asparagine, and increases the rate of glutaminase action. After hydrolysis, which is complete in thirty minutes, the ammonia formed is determined by direct nesslerization or by nesslerization after distillation, depending on the accuracy required. The glutamine content of a number of materials was reported. The glutamine in normal human plasma (6 to 10 mg. per 100 cc.) constitutes about 20 per cent of the total  $\alpha$ -amino nitrogen of the plasma. This points to glutamine as of importance in the intermediary metabolism of nitrogen (12). Glutaminase, asparaginase, and  $\alpha$ -amino acid deaminase are absent from plasma, while nucleoside deaminase and either phosphatase or nucleotide deaminase are present in plasma (13).

*Urease.*—The inhibition of urease by ascorbic acid (14), which Quastel (15) suggested might be due to dehydroascorbic acid, was shown by Giri & Seshagiri Rao (16) to be caused by neither the oxidized nor the reduced ascorbic acid but to be associated with the oxidation of ascorbic acid by traces of copper. Compounds, such as cysteine, that inhibit ascorbic acid oxidation annul the inhibition of urease by the vitamin. The inhibition may be due to an intermediate oxidation product such as cuprous oxide (16) or perhaps due to hydrogen peroxide, which is produced in the chemical oxidation of ascorbic acid (17).

The inhibition of urease by partially purified preparations of penicillin (18) must be attributed to impurities, since crystalline penicillin does not inhibit urease (19).

Sumner & Sisler (20) recommend the use of crystalline urease

for urea determinations rather than urease purified by other means (21). Directions for the crystallization of urease are repeated (20) and it is emphasized that urease is easy to crystallize and does not need to be recrystallized to remove residual canavanine or arginase for use in blood urea determinations. Urease crystals from 200 grams of "Arlco" jack bean meal will suffice for 11,500 blood urea analyses. Van Slyke & Archibald (22) reported carefully worked out procedures for measuring urease activity. Besides the manometric determination of carbon dioxide and the titrimetric determination of ammonia, a third method described is based on the colorimetric measurement of the speed of the pH increase (methyl red indicator) in a specified phosphate-urea solution.

Weil (23) found that urease activity is present in red blood cells and that the activity is increased by autolytic processes. The red blood cell urease is inhibited by plasma, thus permitting the accumulation of urea in plasma. Kidney, gastric mucosa, pancreas, brain, thymus, muscle, and Philadelphia No. 1 sarcoma of rat do not exhibit urease activity.

#### ESTERASES

*Cholinesterases.*—Although the characteristics of the two or more enzymes that can hydrolyze acetylcholine differ in a number of ways, it appears that they can be differentiated most suitably by their specificity (24). Classification according to their locale is not sufficient; for example, sera, depending on the species, contain nonspecific cholinesterase predominantly, a high proportion of specific cholinesterase, or only specific cholinesterase. With specificity as a primary criterion it is not always true that the activity of the specific enzyme, as contrasted with the activity of the nonspecific enzyme, decreases as the substrate concentration is increased. Thus *Planaria* contains a specific cholinesterase that is not inhibited at high substrate concentrations. Furthermore, in the presence of clupein sulfate the activity of the specific enzyme increases with increasing substrate concentration (24). In spite of objections (25) Mendel & Rudney (24) wish to use the prefix "pseudo-" to emphasize the nonspecific character of the enzyme that hydrolyzes esters other than choline esters.

The inhibition characteristics of the two types of cholinesterase not only emphasize their individuality but provide a means of studying their physiological function. Zeller & Bissegger (26) demonstrated that human serum cholinesterase activity, which is largely due to the

nonspecific enzyme, is inhibited by nupercaine, irgamide, isopropyl-antipyrine, and caffeine, whereas the esterase activities of erythrocytes and brain are only weakly inhibited by these compounds. Morphine inhibited the esterase from all three sources. These authors concluded from the inhibition studies and other results, as did Mendel *et al.* (27), that two types of cholinesterase exist. Harris & Harris (28) reported that the very active inhibitor in curare, which, they found, specifically inhibits the nonspecific esterase, cannot be any of five pure curare alkaloids that were tested. Roca & Llamas (29) noted that the cholinesterase activity of serum was not inhibited by thiamine but was inhibited slightly by yeast extract containing phosphorylated thiamine and strongly by cocarboxylase. The inhibition of horse serum cholinesterase activity was proposed (30) as a method for estimation of physostigmine at the very low concentrations of  $10^{-8}$  to  $10^{-6}$  *M*. Ellis *et al.* (31) showed that eseroline, a product of the hydrolytic cleavage of physostigmine, does not inhibit horse serum cholinesterase activity, whereas rubreserine and eserine, which are formed from eseroline, do inhibit, though with a hundredth the potency of physostigmine. Distinction between the cholinesterases was not made in this study.

Strelitz (32) purified pseudocholinesterase from horse serum about 5,000-fold as compared with previous purifications of serum enzyme of 150-fold. The enzyme was more stable than a purified preparation of high specific activity made from dog pancreas (33). However, like the preparation from dog pancreas, dilution of the purified serum enzyme caused inactivation except in the presence of stabilizing factors, such as gum acacia. Although the purified serum enzyme hydrolyzed tributyrin and other esters, all of the tributyrinase activity of the serum was not due to the pseudoesterase, because serum hydrolyzed tributyrin at 140 per cent of the rate that it hydrolyzed acetyl- $\beta$ -methylcholine, whereas purified enzyme hydrolyzed tributyrin at only 30 per cent of this rate. The maintenance of the relative rate of 30 per cent through several steps of the latter stages of purification indicates that the ability to hydrolyze other esters is a characteristic of the horse serum cholinesterase and Strelitz offered this as evidence that the enzyme should be classified as "pseudo-cholinesterase." Although inhibition tests were not made with the purified enzyme, physostigmine did not inhibit the tributyrinase activity of serum, though it did inhibit the acetyl- $\beta$ -methylcholine activity (27). If the two activities are attributes of the same enzyme there would appear to be more than one "active group" or mechanism of combination of enzyme with its sub-

strates. Bader *et al.* (34) obtained a crystalline serum mucoprotein that had about twenty times the activity of serum, on a dry-weight basis. As the authors point out, the reason for the big discrepancy between their preparation and that of Strelitz (32) is not clear. In the purification of the mucoprotein some impurities were removed by a foaming technique. The enzyme, in contrast with impurities, remained in the solution at pH 8 (the condition used to effect purification) but was adsorbed on the foam at acid pH values.

By using the specific substrates for true and pseudocholinesterase, Mundell (35) showed that the greater activity of the plasma of mature female rats toward acetylcholine (previously reported by other workers), compared with that of mature males and immature females, is due mainly to the activity of the pseudoesterase. Nachmansohn & Rothenberg (36) found that nervous tissue, unlike other tissue, contains essentially only the specific cholinesterase.

Purified cholinesterases have been used in physiological studies by Mendel & Hawkins (37). They showed that intravenous injection of pseudocholinesterase prevented chromodacryorrhea (shedding of reddish tears) caused by subcutaneously injected acetylcholine. The injection of either pseudo- or true cholinesterase (six to seven times as many units of the pseudocholinesterase was used because of its lower rate of action at low substrate concentrations) prevented reflex response of the rat pupil to light. This is strong evidence that acetylcholine plays a role in the reflex response of the pupil, though it is not known whether it is effective at the synapses in the retina, the central synapsis in the oculomotor nuclei, the synapses in the ciliary ganglion, or the endings of the short ciliary nerves on the sphincter pupillae. By histological and chemical studies, Anfinsen (38) compared the cholinesterase activity of sections of bovine retina rich in synaptic material with sections containing few or no synaptic structures. The predominantly synaptic localization of the cholinesterase activity that was found provides more direct support than has been provided by less critical histological studies for the theory of chemical mediation of the nervous impulse at the synapses.

*Lipase.*—The energy of activation (Arrhenius equation) for pancreatic lipase was reported to be about 9,000 cal. at high concentrations (*ca.* 0.1 *M*) of the triglycerides of caproic, heptylic, and caprylic acids and about 20,000 cal. or more at low concentrations (*ca.* 0.002 *M*) of these esters (39). However, only small differences were observed for tributyrin and trivalerin at high and low concentrations.

It would be of interest to test the suggestion that combination of certain, if not all, of the triglycerides with the enzyme is endothermic, by making dissociation constant ( $K_m$ ) determinations at different temperatures. The complexity of the lipase system is indicated again in a report (40) showing that pancreatic lipase was not activated at pH 4.7 by calcium chloride or sodium glycocholate, whereas it was activated at pH 8.9. Furthermore, the activation by the several substances tested depended on the substrate used as well as on the pH.

The hydrolysis of castor oil by Ricinus lipase is stimulated by cations generally and inhibited by anions (41). The apparent effects of the ions were small (less than 25 per cent change), possibly because the controls showed a high percentage of hydrolysis (*ca.* 75 per cent).

*Pectinesterase (pectase).*—This enzyme occurs in sufficient quantities in citrus fruit peel to de-esterify pectin rapidly when a slurry of the peel is maintained at pH 7.5 or above (42). The characteristics of this apparent de-esterification *in situ* were similar to the action of enzymes in general in homogeneous solution to the extent that the activity varied with the pH in the usual manner and the energy of activation was about 6,000 cal. for the enzymic de-esterification, compared with 11,000 cal. for the alkaline de-esterification of pectin. Since at pH 8 about 25 per cent of the citrus esterase is soluble (43), whereas the albedo pectin is insoluble under these conditions, the de-esterification in the slurry might have been produced largely by the action of dissolved enzyme on undissolved substrate.

The actions in homogeneous solutions of pectinesterases from alfalfa (44), citrus fruit (43), and similar plant, but not fungal, sources were recently found to be increased markedly, yet unspecifically, by cations. The increase in activity, as is the case with many enzymes, depends on the pH; thus the activity was increased about thirty-fold at pH 5.5 by the addition of sodium chloride to 0.15 *M* but was only slightly affected at pH 8.5. Curves relating enzyme activity and pH were shifted to lower pH regions as the cation concentration was increased. Divalent cation salts were more effective than monovalent cation salts even at equal ionic strengths. In the presence of a suitable electrolyte concentration, the esterase had practically full activity over the pH range 4.5 to 9 (43). The results are consistent with a mechanism of action of cations based on prevention of inhibition rather than on activation (45). Thus it was postulated that (*a*) essentially cationic enzyme (pH 7 or less) forms an inactive combination with pectin or

pectic acid carboxyl groups (pectic acid inhibits at pH 6), (b) essentially anionic enzyme (pH 8.5 or more) does not combine with the carboxyl groups (pectic acid does not inhibit at pH 8.5), and (c) at the lower pH values cations, by competition, dissociate the inactive complex and increase the enzyme activity (pectic acid inhibition at pH 6 is decreased by cations). It is implicit in these postulates that the ionic forms of the enzyme at the lower and higher pH values, if uncombined with carboxyl groups, have practically equal activities.

### GLYCOSIDASES

*Amylases.*—Improved means of obtaining  $\alpha$ - and  $\beta$ -amylases free from each other have been reported. Kneen *et al.* (46) found that calcium ion is an instability factor for  $\beta$ -amylase, as well as a stability factor for  $\alpha$ -amylase, and thereby improved the Ohlsson (47, 48) procedure for preparing  $\alpha$ - and  $\beta$ -amylase from malt. Barley malt extracts were freed of  $\beta$ -amylase, with very little loss of  $\alpha$ -amylase, by heating the extract at pH 6 to 7 and 70° C. for fifteen minutes in the presence of calcium ion (*ca.* 0.05 per cent).  $\alpha$ -Amylase was destroyed, with little loss of  $\beta$ -amylase, by holding dialyzed (calcium-free) extracts at pH 3.0 and 30° for two hours. The cereal amylases may also be obtained nearly free of each other by selection of suitable source material.  $\beta$ -Amylase, of course, can be obtained nearly free of  $\alpha$ -amylase from ungerminated cereal, while  $\alpha$ -amylase can be obtained from germinated oats, maize, or sorghum, which Kneen (49), in a comparative study of the development of amylases in germinating cereals, found to contain less  $\beta$ -amylase than could be detected by the customary assay method. Laufer *et al.* (50) reported that the amylase ( $\beta$ -amylase) in soybean seed is "free," and that germination does not result in the production of more than a trace of  $\alpha$ -amylase. Soybean has been used as a source of  $\beta$ -amylase and its properties studied (51).

Use has been made of amylases in the characterization and determination of starch and glycogen. Morris (52) obtained only 20 per cent hydrolysis of corn glycogen by wheat  $\beta$ -amylase, which indicates greater branching than in rabbit glycogen (45 per cent hydrolysis) (53), starches (60 to 70 per cent hydrolysis), or in unbranched amylose (100 per cent hydrolysis). Kuzin & Makaeva (54) described a micromethod, which uses malt amylase, for the determination of glycogen in blood and tissue. Polysaccharides other than glycogen were not hydrolyzed by the enzyme preparation. Myers *et al.* (55)

have modified Myers' animal diastase method, which was designed originally for whole blood, so that it can be used for determining serum or plasma diastase.

$\alpha$ -Amylase not only liquefies, dextrinizes, and slowly saccharifies boiled starch, but it also hydrolyzes native starch (56). Balls & Schwimmer (57) obtained almost complete conversion of raw starch to glucose (predominantly) and maltose with a mixture of enzymes from pancreas and *Aspergillus oryzae* ("mold bran"), which contain  $\alpha$ -amylase. Calcium chloride, calcium nitrate, sodium chloride, or the ash of wheat flour promoted rapid digestion. The chloride and nitrate ions, of course, would accelerate the action of the pancreatin. The speed and extent of hydrolysis of the raw starch were greater than those obtained by Stamberg & Bailey (58), who obtained as much as 20 per cent hydrolysis by the action of comparatively small amounts of malt  $\alpha$ -amylase on raw starch at 30°. Balls & Schwimmer, on the other hand, obtained 83 per cent conversion of 3.2 gm. of raw starch at 40° in four hours by the simultaneous action of 1.0 gm. of *Aspergillus* amylase and 0.1 gm. of pancreatin in 100 ml. of a solution that was 0.05 *M* in acetate buffer (pH 5.2) and 0.03 *M* in calcium chloride. The rate of digestion of raw starch decreased markedly with increasing extent of digestion as is the case also with cooked starch. Presumably, at least in part, this is because the dextrans are attacked more slowly. However, in view of the influence of calcium on the stability of malt  $\alpha$ -amylase (46), part of the decrease, especially in the absence of calcium, might have been due to enzyme inactivation. Thus in the saccharification of starch by kaffir malt (*Sorghum vulgare*) the increased conversion caused by calcium ion was attributed, though without experimental confirmation, to stabilization of the enzyme (59). Raw wheat starch was more rapidly digested than raw potato starch by the pancreatin-*Aspergillus* amylase mixture (57), as was found previously to be the case with other amylases by Lynst-Zwicker according to Stamberg & Bailey (58). Although the rate of action of the enzymes on cooked starch was faster than on raw starch, neither a comparison of the whole course of digestion nor a comparison of the initial rates of action on the cooked and raw starches has yet been made.

Bowman (60, 61) obtained from navy beans by thorough ether extraction an oil that markedly inhibits the digestion of starch by pancreatic amylase. The variation in inhibition with kind of starch suggests an inhibitor-starch interaction rather than an inhibitor-

enzyme interaction. The inhibitor appears to differ from the protein-like inhibitor present in grain (62).

An understanding of the enzymic hydrolysis of starch requires a knowledge of the starch structure, of the specificity of  $\alpha$ - and  $\beta$ -amylases, of the possible action of concomitant polysaccharidases in the  $\alpha$ - and  $\beta$ -amylase preparations, of the structure of the dextrans formed, and of synthetic reactions that may occur. The following reports concerning amylase action are consistent with a starch structure of maltose and isomaltose linkages between glucose residues, and they give some information about the structure of the dextrans and about synthetic reactions during starch hydrolysis; however, they are inconclusive with regard to concomitant polysaccharidases.

The hydrolysis of arrowroot starch (63) by malt  $\alpha$ -amylase (prepared according to Ohlsson) was similar to the hydrolysis of potato, barley, and corn starches. The first stage of hydrolysis, which yielded  $\alpha$ -dextrans, proceeded twenty to fifty times more rapidly than the second stage, saccharification (64). The rate decreased sharply at about 16 per cent hydrolysis of the glycosidic bonds of these starches (65, 66). With amylose the decrease in rate occurred at about 22 per cent hydrolysis. From a study of the enzymic hydrolysis of a number of various sized polysaccharides prepared by acid hydrolysis of starch, Myrbäck & Thorsell (67) concluded that the occurrence of  $\alpha$ -amylase hydrolysis of starch in two stages depends on the size of the polysaccharide intermediates and on the nature of the enzyme-substrate combination rather than on the kind of glycosidic bonds in the intermediates, i.e., other than the isomaltose bonds already known to be present. However, this does not appear to exclude the possibility that other polysaccharidases in  $\alpha$ -amylase preparations may play a part in the saccharification, though it is generally considered that slow hydrolysis of the  $\alpha$ -dextrans is catalyzed by  $\alpha$ -amylase (68, 69). That the  $\alpha$ -dextrans, which were not of uniform molecular weight, possessed different kinds of glycosidic bonds (different distribution of 1,4- and 1,6-glycosidic linkages) was indicated by the observation (63) that  $\alpha$ -dextrin fractions with molecular weights near 1200 (the "normal structure" dextrans, containing 6 to 8 glucose residues) were almost completely saccharified by  $\beta$ -amylase, whereas the fractions with molecular weights over 1300 ("abnormal structure," i.e., branched) were incompletely saccharified. Likewise, saccharification by  $\alpha$ -amylase approached completion more rapidly with dextrin



fractions of lower molecular weights. The similar results obtained in the dextrinization of the various starches (potato, corn, rice, arrow-root, wheat, and barley), as well as in the saccharification of the  $\alpha$ -dextrins, were cited by Myrbäck *et al.* (70) as indicating that the relative number of anomalies (i.e., branchings, the length of the side chains, etc.) in these starches are nearly the same.

Stark (71) showed that the formation of unfermentable limit or residual dextrins by amylase was due at least in part to the reversion of fermentable sugars to larger, nonfermentable compounds. The fact that the extent as well as the rate of conversion to maltose and glucose was affected by the starch-to-enzyme ratio, and probably also by the temperature, pH, and other factors, gives rise to the question: to what extent is the limited conversion due to enzyme inactivation, as was postulated in one case (59), to equilibrium, or to a stoichiometric factor added with the enzyme? Thus comparative studies of limit dextrins are complicated. Myrbäck *et al.* (70, 72) found that arrow-root and barley starch digested with malt amylase yielded about 5 per cent limit dextrins, whereas digestion with salivary amylase yielded several times as much limit dextrins. The latter limit dextrins, which were of higher molecular weight than those obtained with malt, could be hydrolyzed in the presence of large amounts of various amylase preparations. Conversion to fermentable sugars was most rapid and complete with takadiastase. The fermentable sugars produced by takadiastase and by pancreatin consisted chiefly (about 90 per cent) of glucose, whereas  $\alpha$ -amylase and salivary amylase, which acts on its own purified limit dextrin, produced chiefly maltose. These observations indicate that the limit dextrins are split in part, if not entirely, by carbohydrases other than the amylases, unless, as is possible, the amylases from different sources differ in specificity. The hydrolysis of salivary limit dextrins by salivary amylase, of course, also indicates that equilibria are involved in addition to limited hydrolysis and synthesis.

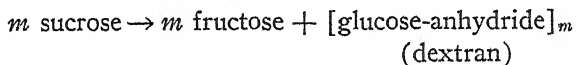
Pigman (59) studied the synthetic action of amylase preparations and equilibria that may be responsible for incomplete conversion of starch to fermentable sugars. To simulate commercial practice, he used 10 to 13 per cent starch concentrations. The observation that mixtures of several amylase-containing emulsins frequently resulted in the production of larger rather than smaller amounts of limit dextrins led him to investigate the synthesizing action of amylases. Maltose, but not dextrose, was converted 25 per cent or more into

unfermentable sugars by fungal, bacterial, and pancreatic amylases, whereas malt amylases, and wheat and soybean  $\beta$ -amylase did not produce fermentable sugars from maltose. When the fungal enzymes were present during fermentation, the fermentable materials were completely reconverted to fermentable materials, but when the bacterial and pancreatic enzymes were present the fermentable materials were only partially reconverted. The synthesis from maltose of fermentable sugars by the fungal, bacterial, and pancreatic enzymes, but not by malt amylases, again emphasizes the complexity of the problem of elucidating the starch degradation mechanism with enzyme mixtures. These results on the synthesis of fermentable sugars might be expected with a mixture of enzymes in view of the synthesis of dextrans and levans from sucrose (see below) and of Schardinger dextrans by *Bacillus macerans* amylase (73). Also the presence of phosphate in the reaction mixture would permit the action of phosphorylases, if they were present in the enzyme material used (59).

The cereal  $\alpha$ -amylases and *Aspergillus* amylase were able to convert starch practically completely to fermentable sugars when they were allowed to act on starch in the presence, but not in the absence, of yeast (59). Thus synthesis of nonfermentables was prevented by removal of the reactants forming them by fermentation. The incomplete conversion in the absence of yeast appeared to be due, at least in part, to an equilibrium between fermentable and fermentable sugars, since the fermentable sugars were further hydrolyzed by these enzymes after removal of the fermentable sugars (*cf.* Myrbäck *et al.* 72). Pancreas and *Bacillus mesentericus* enzymes differ from these enzymes in that they fail to hydrolyze starches completely to fermentable sugars even in the presence of yeast. To what extent these differences are due to differences in the character of the  $\alpha$ - and  $\beta$ -amylases in these preparations and to what extent they reflect the presence of other saccharidases is not known.

*Dextranucrase and levansucrase.*—The names “dextranucrase” and “levansucrase” were proposed for the enzymes synthesizing dextran and levan from sucrose (74, 75) to replace Beijerinck’s term “viscosaccharase.” These enzymes, which have only recently been prepared and studied in cell-free and particle-free solution, catalyze *in vitro* the synthesis of dextrose-containing and levulose-containing polysaccharides that differ from the starch and glycogen formed by the action of phosphorylase on glucose-1-phosphate. Hehre & Sugg

(76, 77) showed that dextran synthesized by sterile cell-free enzyme from *Leuconostoc mesenteroides* was similar chemically and serologically to the dextran produced by the bacteria. The synthesis occurred in the absence of appreciable amounts of phosphate, did not occur with glucose-1-phosphate, nor was dextran formed from sucrose by potato phosphorylase (78). Therefore, the synthesis of dextran is similar to that of starch and glycogen only to the extent that the substrates are glycosides and the product, which differs in many ways from starch and glycogen, is a glucose-containing polysaccharide. The dextran synthesis, in which all the sugar is accounted for, appears to proceed as follows (76):



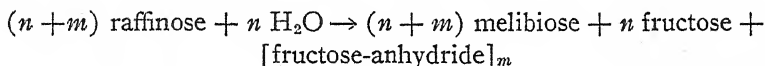
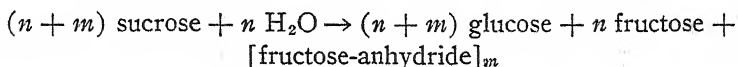
Stacey (79) found much higher yields of the exocellular dextran-sucrase in the culture liquor from *L. mesenteroides* and *Saccharomyces cerevisiae* grown in symbiotic association than in pure cultures of *L. mesenteroides*.

Levansucrase was prepared in cell-free form from *Bacillus subtilis* and *Aerobacter levanicum* (75, 80). The enzyme is formed adaptively in *B. subtilis*, whereas it is constitutive in *A. levanicum*. On sucrose agar media the *B. subtilis* enzyme is exocellular, whereas the *A. levanicum* and *Bacillus polymyxa* enzymes are endocellular. The polysaccharide formed by the action of the *A. levanicum* enzyme was shown to be a levan, but its identity with levans from other sources was not demonstrated (75).

The mechanism of levan production by *A. levanicum* levansucrase has been elucidated by Hestrin & Avineri-Shapiro (74, 81). Levan was produced from sucrose and more slowly from raffinose, but not from fructose and other hexoses, trehalose, maltose, lactose, inulin, methylfructofuranoside, glucose-1-phosphate, fructose-1,6-diphosphate, or fructose-6-phosphate. The enzyme thus appears to require a sucrose unit in its substrate, although the glucose moiety can be substituted as in raffinose. The failure of melizitose to serve as substrate for levan production by living cells of *B. subtilis* indicates that an unsubstituted fructose moiety may be essential in the substrate. The individuality of levansucrase, indicated by its specificity, is supported by the fact that this enzyme differs from both yeast fructosaccharase and phosphorylase in susceptibility to sugar inhibitors. Furthermore,

Hestrin (82) showed that the synthesis of levan does not arise from the fructofuranose formed in the enzymic hydrolysis of sucrose or raffinose. Addition of invertase to the levansucrase system did not increase the rate of levan synthesis with high or limiting concentrations of sucrose, nor with raffinose which gives a slow rate of synthesis. On the fructofuranose hypothesis, the slow rate with raffinose would be due to a slow limiting rate of formation of fructofuranose. The fact that levan synthesis does not occur when  $\gamma$ -methylfructoside is hydrolyzed in the presence of levansucrase is also a strong argument against the fructofuranose origin of levan.

Levansucrase catalyzes the formation of levan (fructose-anhydride) as follows (81):



The reaction products represent the total amount of substrate acted upon. The molal quantities  $n$  and  $m$  are nearly equal. In spite of this indication that the aldoses and fructose arise from invertase action, a typical gluco- or fructo-saccharase was not found in the enzyme preparation; nor was any levan-hydrolase found to be present, although living cells of *A. levanicum* ferment levan. The synthesis does not appear to be coupled with an energy-yielding reaction, unless the reaction that yields aldose and fructose is coupled with the synthesis. This possibility is not supported by the observation that, in dextran formation, all of the glucose from the utilized sucrose appeared as dextran. The substrate for both dextran and levan synthesis is a glycosidic derivative that, like glucose-1-phosphate, possesses a higher energy content than do simple hexoses (77, 81).

$\beta$ -Glucuronidase.—An enzymatic method for the hydrolysis of sodium pregnanediol glucuronide in preparation for the analysis of the hormone was developed by Talbot *et al.* (83). The partial destruction of the pregnanediol by hydrochloric acid hydrolysis was thus avoided. Although the beef spleen  $\beta$ -glucuronidase (84, 85) did not give as satisfactory results as an enzyme preparation made from acetone dried rat liver, the effective rat liver enzyme must be also a  $\beta$ -glucuronidase, since Huebner *et al.* (86) showed by synthesis that the naturally occurring glycoside is pregnanediol 3- $\beta$ -*D*-glucuronide.

## MISCELLANEOUS ENZYMES

*Thiamine-destroying enzyme.*—The identification and isolation of the reaction fragments (87) 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine, proved, as previously indicated (88), that the inactivation of thiamine by a heat-labile factor in fish tissue is enzymic rather than stoichiometric as, for example, is the inactivation of biotin by avidin. Although the over-all reaction is a simple hydrolysis, it appeared that an intermediate was formed that was changed readily to the final product in tissue suspensions, but was changed only slowly in sodium chloride extracts. The enzyme extracted by 10 per cent sodium chloride consists of a heat-stable, dialyzable component, in addition to the heat-labile part. In confirmation of the above mechanism, inactivation of thiamine was found to be accompanied by the liberation of a hydrogen ion, as required for the transformation of the quaternary nitrogen salt to a tertiary nitrogen (89).

The enzymic destruction of thiamine was inhibited markedly by cupric, zinc, and ferric ions, to some extent by cyanide, fluoride, iodoacetate, sulfhydryl and sulfite, and strongly by *o*-aminobenzyl- and  $\beta$ -aminoethyl-4-methylthiazolium chlorides, which resemble thiamine (90). Substitution of the amino group in either of the last named inhibitors abolished or decreased the inhibition. Nonthiazole pyrimidine derivatives did not inhibit as markedly as did the unsubstituted aminothiazole derivatives. Thus, inhibition depends to a considerable extent on the amino group attached through carbon to the thiazole ring. The Michaelis constant for thiamine was  $0.83 \times 10^{-4} M$  and the inhibition constant for *o*-aminobenzyl-4-methylthiazolium chloride, which was shown to inhibit competitively, was  $0.020 \times 10^{-4} M$ .

*Carbonic anhydrase.*—The specific activities and the zinc contents of two purified preparations of carbonic anhydrase were reported to differ. In reply to Keilin & Mann (91) who reported that their preparation contained 0.30 to 0.33 per cent zinc, Scott & Fisher (92) contend that the zinc content of their carbonic anhydrase preparation was near 0.20 per cent and cited a polarographic determination that gave 0.22 per cent zinc. They suggested that the difference in zinc content of the preparations from the two laboratories was real and was not to be attributed to a difference in experimental methods. The difference in specific activity of the two preparations was explained on the basis of experimental method (91) but was not submitted to test.

It was shown that carbonic anhydrase activity in red blood cells is inhibited specifically by small amounts of sulfanilamide and that the activity in the cells is linked with the acid-base changes in the plasma (cf. *Annual Review of Biochemistry*, Vol. 13). Benesch *et al.* (93) reported that hens fed subpoisoning doses of sulfanilamide produced shell-less eggs even though supplied with adequate calcium, whereas the same hens when fed sulfapyridine, which does not inhibit carbonic anhydrase, produced eggs with normal shell. Thus the sulfanilamide evidently interfered with normal anion production, i.e., with the acid-base changes in the oviduct. It will be recalled, however, that the production of carbonate in pancreatic juice, which had been attributed to carbonic anhydrase activity, was not inhibited by sulfanilamide (94).

Ashby & Chan (95) have further modified the procedure of Philpot & Philpot for the determination of carbonic anhydrase. In determining the enzyme activity of tissue, the finely ground whole tissue was employed in place of extracts, and a correction was made for the activity due to the blood remaining in the tissue. The blood content was determined by the benzidine reaction. The carbonic anhydrase content of tissue, as distinguished from that of blood, was thus readily determined (96, 97, 98). Carbonic anhydrase activity was not found in adrenal glands. Variable enzyme activity (1 to 28 per cent of that of blood) was found in liver, striated muscle of man, and the medulla of the kidney, whereas fairly constant activity (7 to 14 per cent of that of blood) was found in the central nervous systems of eight species tested. From the pattern of quantitative distribution of carbonic anhydrase in the central nervous system it was postulated that this enzyme might play a part in determining the speed with which energy is made available for nerve impulse conduction (98, 99, 100).

*Mucinolytic enzymes—diffusing factors.*—Hahn (101) purified bull testicular mucinase (hyaluronidase) 10,000-fold and found that the mucinolytic activity approximately paralleled the activity in increasing diffusion. This strongly indicates that testicular mucinase possesses diffusion-factor properties. An enzyme preparation that was purified 2,000-fold by ammonium sulfate fractionation and dialysis did not show homogeneous electrophoretic behavior, nor did samples purified a further five-fold appear to be homogeneous on sedimentation in the ultracentrifuge. The active substance was isoelectric at pH 5.7.

A further contribution concerning the part played by sperm hyal-

uronidase in fertilization has appeared. Rowlands (102) reported that a sperm-free filtrate of rabbit semen added to dilute semen increased fertilizing power. This was presumed to be due to the hyaluronidase contained in the sperm-free filtrate, since mucinolytic enzyme preparations liquefy the gel surrounding the ovum. It would be of considerable interest to add purified enzyme, such as Hahn prepared, to the dilute semen in order to avoid the addition of other factors in the sperm-free filtrate.

*Lysozyme*.—This enzyme, which lyses cells of a number of microorganisms, apparently by hydrolyzing the mucopolysaccharide in their structure, has now been crystallized from egg white in high yields (85 to 90 per cent) by a procedure that reproducibly leads to the crystalline product (6). Previous investigators did not report yields, nor was sufficient crystalline material obtained for chemical examination (103, 104). Furthermore, since the assay method is not uniform in different laboratories it is difficult to make comparisons of activities. This difficulty can be surmounted by the use of egg white, which was found (6) to have remarkably constant activity, as a reference standard. Alderton *et al.* (6) prepared lysozyme by adsorbing it directly from egg white on bentonite, eluting inactive proteins in phosphate buffer, and eluting the active protein at pH 5 in pyridine-sulfuric acid solution. Crystallization could be induced at pH values ranging from 3.5 to 10.8. The crystal forms appeared to vary with the pH and with the ions present in solution. The activity of the eluted lysozyme was the same as the activity of the crystalline lysozyme. The purity of the preparation was indicated by electrophoretic and sedimentation studies and by failure of salt fractionation or proteolytic enzymes to remove inactive protein. Earlier work indicated that lysozyme is a basic protein of relatively low molecular weight that is fairly stable to heat in acid solution but is inactivated by heat in alkaline solution (104). Alderton *et al.* found the same properties for their purified lysozyme. The isoelectric point is between pH 10.5 and 11 and the molecular weight is about 17,000. Lysozyme, which represents about 3 per cent of the egg white protein, appeared to be identical with the globulin called G<sub>1</sub> by Longworth *et al.* (105).

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# THE CHEMISTRY OF THE CARBOHYDRATES

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## SUGARS FROM NATURAL SOURCES

A sugar named diginose is obtained by acid hydrolysis of the glycoside diginin from *Digitalis purpurea*. It is 3-methyl-2,6-dideoxy-D-glucose and differs from cymarose only in the stereochemical configuration of the methoxyl group on position 3. Oxidation with permanganate provided evidence for this structure (1) since diginose yielded D-(+)-methoxysuccinic acid and cymarose changed to the L-(—)-isomer.

That digitalose is 3-methyl-6-desoxy-D-galactose is established in recent work (2). In contrast to older statements, the sugar does form an osazone still retaining the methoxyl. Hence, the position of the methyl group on position 2 becomes untenable. Both D-ribose and L-lyxose are present in yeast nucleic acid, with ribose predominating (3). This is apparently the first time that L-lyxose has been found in naturally occurring material. Characterization of these two pentoses was by their crystalline benzimidazole derivatives.

Inulin, known to be present as the polysaccharide in dahlia tubers or in the Jerusalem artichoke, has now been found to be present in the rubber-producing plant guayule (4). Methylation and hydrolysis of the polysaccharide yielded 3,4,6-trimethylfructose.

Karaka kernels contain the glucoside karakin which hydrolyzes to an amino derivative of glucose or mannose and to hiptagenic acid (5). The latter is considered to be glyceraldehydic acid oxime,  $\text{HON}=\text{CHCHOHCOOH}$ . The confusing literature regarding volomitol, the heptitol present in the fungus *Lactarius volemus*, has been clarified by Maclay, Hann & Hudson (6). These investigators also prepared this D-glycero-D-talo-heptitol by hydrogenation of D-mannoketoheptose with Raney nickel catalyst.

Miller (7) found that tobacco plants, in contact with chloral hydrate, brought about its reduction to trichloroethanol by proving that the roots accumulated trichloroethyl  $\beta$ -gentiobioside and the leaves, on a dry basis, contained 13 per cent of a mixture of the  $\beta$ -gentiobioside and  $\beta$ -glucoside.

## SYNTHESIS OF UNCOMMON CARBOHYDRATES

*Glycerose*.—A 67 per cent yield of glycerose was realized by treatment of xylan (from straw) with periodic acid in the presence of sodium acetate as buffer (8).

*Threose*.—Lake & Glattfeld (9) converted D,L-threonolactone to threonamide and then to tribenzoylthreonamide. This amide was converted to the acid by means of nitrogen trioxide and acetic acid, making use of the procedure of Hurd & Sowden (10). Thionyl chloride converted this acid to tribenzoylthreonyl chloride which in turn was changed by hydrogen and palladium, then debenzoylated, to D,L-threose, a syrup.

Tartaric acid is a good source of L-threonic acid (11). Dibenzoyltartaric anhydride, prepared by interaction of benzoyl chloride and tartaric acid, may be reduced in dioxane solution by hydrogen and palladium to dibenzoyl-L-threonolactone from which L-threonic acid or its lactone is obtainable.

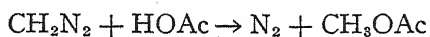
*Ribonic acid derivatives*.—A remarkable cation effect is shown in the conversion of cadmium D-ribonate to tetracetyl-D-ribonic acid by the use of acetic anhydride and hydrogen chloride. The yield was 85 per cent, compared with yields of 4, 22, 25, and 46 per cent from the barium, calcium, potassium, and ammonium salts, respectively (12).

*D-Altronic acid*.—Enzyme treatment (Pectinol 46 AP) of citrus pectin yielded D-galacturonose and this was converted by lime water to 5-oxo-L-galactonic acid in the same type of change as that of glucose into fructose. Hydrogenation by means of Raney nickel catalyst (2300 pounds pressure of hydrogen, 80° C.) of the 5-oxo-L-galactonic acid yielded a mixture of L-galactonic and D-altronic acids which were separated by crystallization of the calcium or cadmium salts (13). This is a convenient synthesis of D-altronic acid.

*Xylitol*.—A new crystalline modification of xylitol, m.p. 93–94°, has been announced (14). The previously noted xylitol melting at 61° is a metastable modification which changes on standing in the air for a few days to the 93° variety. Either form can be obtained directly from alcohol by "seeding" with the desired crystals.

Several new pentaacylxylitols have been reported (15) ranging from the pentapropionates to the pentastearates. Saponification of the higher esters by potassium hydroxide is much faster in boiling butanol than in boiling ethanol.

*keto-D-Sorbose pentaacetate*.—In a reaction general for acid chlorides tetraacetyl-D-xylonyl chloride ( $\text{RCOCl}$ ) reacts with diazomethane ( $\text{CH}_2\text{N}_2$ ) in ether to form tetraacetylxylyldiazomethane ( $\text{RCOCHN}_2$ ). Refluxing this substance with acetic acid changes it to tetraacetylxylylmethyl acetate ( $\text{RCOCH}_2\text{OAc}$ ), just as diazomethane itself would have given rise to methyl acetate:



Since tetraacetylxylylmethyl acetate is simply *keto-D-sorbose* pentaacetate,  $\text{AcOCH}_2(\text{CHOAc})_3\text{COCH}_2\text{OAc}$ , it is obvious that this adaptation of the Arndt-Eistert reaction is a satisfactory method (16) for preparing the open chain modification of sorbose.

#### SYNTHESIS OF DISACCHARIDES

The synthesis of sucrose has attracted the ingenuity of the chemist for a long time, but two stumbling blocks have made the task extraordinarily difficult. One is the furanose ring in the fructose portion of the sucrose molecule, and the other is the fact that both the glucose and fructose units may become attached in  $\alpha$  or  $\beta$  configurations, thereby pointing to several isomeric possibilities in any synthesis.

No unchallenged synthesis of sucrose has yet been achieved by purely chemical methods, but an interesting synthesis by an enzymatic method was developed by Hassid, Doudoroff & Barker (17). These investigators placed glucose-1-(dihydrogen orthophosphate) with fructose in the presence of sucrose phosphorylase from the bacterium *Pseudomonas saccharophila*. Since sucrose was formed directly this reaction medium brings about the essential change of the fructopyranose to the fructofuranose ring.

Coleman *et al.* (18) have been successful in the preparation of  $\beta,\beta$ -trehalose,  $\beta$ -vicianose, and  $\beta$ -primeverose by strictly chemical methods. Trehalose in the form of its octaacetate was prepared by interaction of 2,3,4,6-tetraacetyl- $\beta$ -D-glucose, tetraacetyl-D-glucosyl bromide, and silver oxide. Iodine catalyzes the reaction. Analogous interaction of 1,2,3,4-tetraacetyl- $\beta$ -D-glucose with either triacetyl- $\alpha$ -D-xylosyl bromide or triacetyl- $\beta$ -L-arabinosyl bromide in the presence of silver oxide and of "drierite" gives rise to the heptaacetates of  $\beta$ -primeverose or  $\beta$ -vicianose in much higher yields than have been previously reported.

## SEPARATION OF SUGAR DERIVATIVES BY ADSORPTION

The chromatographic adsorption method for the separation of sugar mixtures has been extended considerably by the work of Coleman *et al.* (19). In this method the sugars are converted to their phenylazobenzoyl esters [glucose,  $C_6H_7O(OH)_5$ , to glucose penta-phenylazobenzoate,  $C_6H_7O(OCOC_6H_4N=NC_6H_5)_5$ ; or cellobiose to cellobiose octaphenylazobenzoate]. A mixture of these esters was separated on a column of silicic acid. From a mixture of  $\beta$ -D-glucose and  $\beta$ -cellobiose, the azoate of glucose, being less readily adsorbed, appears at the bottom of the column and the azoate of cellobiose at the top. After removing the two adsorption bands each was eluted separately with chloroform containing a little alcohol. Several sugar mixtures have been separated in this manner:  $\alpha$ -D-glucose and  $\beta$ -D-fructose,  $\alpha$ -lactose and sucrose, methyl  $\alpha$ -D-glucoside and methyl  $\beta$ -D-cellobioside, and others. One objection to the process in this form is that the azo part of the molecule so greatly outweighs the carbohydrate portion. To meet this objection, Coleman acetylated the sugar and converted it to the acetoglycosyl bromide which, with silver phenylazobenzoate, yielded a molecule containing but one azo group. A mixture of tetraacetyl- $\beta$ -D-glucosyl phenylazobenzoate and heptaacetyl- $\beta$ -cellobiosyl phenylazobenzoate was found to be very easily separable in the silicic acid column. Separation of mixtures derived from 3-methyl-, 2,3-dimethyl-, 2,3,6-trimethyl-, and 2,3,4,6-tetramethylglucose by conversion to the phenylazobenzoates and adsorption on silica has also been reported (20).

Azo functions are not necessary for the successful separation of carbohydrates by adsorption methods. Jones (21) reported that a mixture of methyl trimethylglucoside and methyl tetramethylglucoside was separable in an alumina column, the tetramethylglucoside moving preferentially to the bottom of the column by development with a mixture of ether and petroleum ether. The success of this separation obviously is related to the fact that the free hydroxyl group in the trimethylglucoside gives the compound a greater adsorbing tendency; but it is interesting to note that some separation was achieved when applied to a mixture of methyl trimethyl-L-arabino-furanoside and methyl trimethyl-D-xylopyranoside.

Jones extended these findings to end-group analysis of rice starch and banana starch, by methylation, hydrolysis, and then adsorption to detect the relative quantities of tetra- versus trimethylglucoside pres-

ent, the tetra being indicative of terminal glucose units in the starch molecule. These results showed thirty-three glucose units in rice starch and twenty-six in banana starch.

### OXIDATIONS

Preferential oxidation of the primary alcohol groupings of cellulose by nitrogen dioxide was reported by Kenyon *et al.* (22). In related work, Maurer & Drefahl (23) found that nitrogen dioxide converts galactose to mucic acid (75 per cent yield) and that it oxidizes methyl  $\alpha$ -D-glucoside and methyl  $\alpha$ -D-galactoside to methyl  $\alpha$ -D-glucuronoside and methyl  $\alpha$ -D-galacturonoside, respectively.

Alkaline permanganate was the reagent used to oxidize methyl 2,3,4-trimethyl- $\alpha$ -D-mannoside (prepared from the 6-triphenylmethyl derivative) to methyl 2,3,4-trimethyl- $\alpha$ -D-mannuronoside. The analogous glucose and galactose compounds were oxidized similarly. Esterification of the carboxyl group in this uronic acid by methanol and hydrogen chloride gave rise to methyl 2,3,4-trimethyl-D-manno-saccharaldehyde (24). Helpful details for the purification of galacturonic acid have been reported (25), involving crystallization of the relatively insoluble double salts (Na-Ca, Na-Sr, Na-Ba, Na-Cd, Na-Pb, K-Ca).

2-Oxo acids in the sugar series have been prepared by oxidizing lactones in methanol solution with a mixture of phosphoric acid, sodium chlorate, and a small quantity of vanadium pentoxide (26). Methyl 2-oxo-D-gluconate was made in 60 per cent yield by this method from D-1,4-gluconolactone. Other substances oxidized were potassium D-galactonate, D-glycero-D-gulo-1,4-heptonolactone, and potassium D-glycero-L-manno-heptonate. These 2-oxo acids are, of course, closely related to ascorbic acid.

Many applications of lead tetraacetate and periodic acid as oxidizing agents with carbohydrates have been reported. These will be mentioned below.

### HYDROGENATIONS

4-Methyl-D-mannose was obtained by treating 4-methyl-1,5-mannonolactone with hydrogen at ordinary pressure and temperature in the presence of platinum catalyst (27).

Natural styracitol may be obtained from *Styrax obassia*. Its structure has been a controversial matter for many years, but recent work



appears to have clinched it as 1,5-anhydro-D-mannitol (28). The compound was synthesized from 1,2-glucoseene tetraacetate (made from tetraacetylglucosyl bromide and diethylamine) by hydrogenation with a palladium catalyst. Deacetylation of the tetraacetate to styracitol itself was by sodium methoxide. That styracitol was of the D-mannitol configuration, rather than D-glucitol, was established by oxidation of its tetramethyl ether by nitric acid. Dimethyl-D-tartaric acid was found, but no dimethyl-L-tartaric acid. The latter would have been formed if a D-glucitol structure had been involved. As a matter of fact, 1,5-anhydro-D-glucitol has been shown to be the structure of polygalitol, the epimer of styracitol (29).

Support of Pacsu's (1939) structure for turanose as 3-( $\alpha$ -D-glucosyl)-D-fructose was offered by Hudson (30) who hydrogenated turanose (Raney nickel catalyst) and obtained the same glucosyl-mannitol that was obtained by hydrogenation of the epimer of maltose, 4-( $\alpha$ -D-glucosyl)-D-mannose. Positions 3 and 4 in mannitol are equivalent, hence the latter should also give rise to 3-( $\alpha$ -D-glucosyl)-D-mannitol, thereby establishing the glucosyl attachment in turanose at position 3 with the same certainty that applies for the 4-attachment in maltose.

Reduction of the  $-\text{CH}(\text{SC}_2\text{H}_5)_2$  group to  $-\text{CH}_3$  in 66 per cent yield was realized in the conversion of pentaacetylgalactonaldehyde diethyl mercaptal to 1-desoxy-D-galactitol pentaacetate (31), by refluxing in alcohol solution with Raney nickel. This type of reduction of C-S bonds is an extension of the work of Bougault (1938) and Folker (1943). No flow of hydrogen gas is supplied, but enough hydrogen is occluded within the nickel to cause the hydrogenolysis.

#### REACTION OF SUGARS AND AMINES

The reaction of glucose at 70° with alkylamines (butylamine, pentylamine, heptylamine, octadecylamine, ethylenediamine, etc.) in the presence of a little of the amine hydrochloride gives rise to glucosylalkylamine by exchange of the hemi-acetal hydroxyl of glucose for the amine group. Hydrogenation of these compounds in the presence of Raney nickel opens the oxygen bridge to produce "glucamines,"  $\text{HOCH}_2(\text{CHOH})_4\text{CH}_2\text{NHR}$ , in good yields (32).

Ammonium chloride also catalyzes the interaction of an aqueous solution of glucose with sulfapyridine to yield N-glucosylsulfapyridine (33). Similarly prepared were galactosylsulfapyridine, lactosylsulfanilamide, and *p*-galactosylaminophenyl sulfone. When taken intern-

ally N-galactosylsulfapyridine appears in the blood in higher concentrations (up to 25 per cent) than sulfapyridine itself.

The open-chain compound pentaacetyl-D-gluconaldehyde undergoes cyclization by reaction with ammonium hydroxide (34). N-Glucufuranosylacetamide is formed. The ring size was determined by oxidation with lead tetraacetate.

An intermediate in the synthesis of riboflavin, namely, N-D-ribityl-3,4-dimethylaniline was prepared in the following way: D-ribonolactone was converted to D-ribon-(3,4-dimethylanilide) by heating with 3,4-dimethylaniline. After acetylation of the four hydroxyl groups this anilide (RCONHAr) was changed to its imido chloride (RCCl=NAr) by treatment with phosphorus pentachloride. Hydrogenation of the imido chloride was effected with palladium catalyst (to RCH<sub>2</sub>NHAr). The final step was deacetylation by sodium methoxide (35).

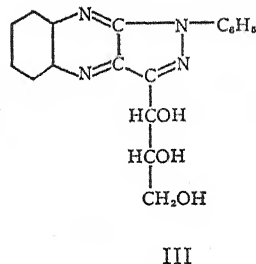
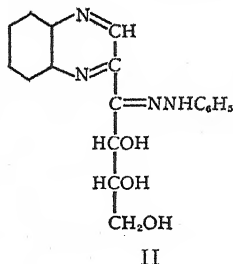
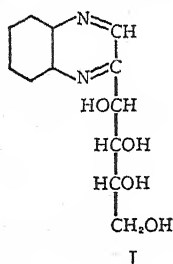
Impregnation of agricultural roughages such as sugar beet pulp, corn silage, etc., with ammonia under pressure causes fixation of the nitrogen, presumably by reaction of the ammonia with the carbohydrate content of the pulps. A palatable and nourishing cattle food results (36).

#### OSOTRIAZOLES AND FLVAZOLES

Experiments with glucose phenylosazone have been performed by Hann & Hudson (37) which become readily understandable when viewed in the light of an experiment with benzil bisphenylhydrazone performed fifty-six years ago by Auwers & Meyer. Aniline was detached when the compound was heated and triphenylosotriazole was the resulting product. (In the 5-membered osotriazole ring the three nitrogen atoms are all adjacent.) In the present work osotriazoles were also formed by detachment of aniline. To do this a solution of the osazone was boiled with dilute copper sulfate solution. 2-Phenyl-4-(D-arabo-tetrahydroxybutyl)osotriazole was formed from glucose. The osazones of galactose, altrose, xylose, cellobiose, lactose, and turanose behaved analogously.

Interest in the flavazoles arises from the fact that aldoses or ketoses are necessary reagents in the formation of this heterocyclic system and that the asymmetry of atoms 2 and 3 disappears in the process. Ohle (38) has been primarily responsible for the development of this field. To make the compounds, o-phenylenediamine and the aldose or ketose are condensed in the presence of phenylhydrazine. The first

step of the process resembles osazone formation, phenylhydrazine serving in its customary role as dehydrogenating agent. From glucose, 2-(*D-arabo*-tetrahydroxybutyl)quinoxaline (I) is the compound



formed. The second step involves another dehydrogenation by phenylhydrazine, then hydrazone formation to II. This is followed by ring closure to III as a third dehydrogenation takes place. The name of III is 1-phenyl-3-(*D-erythro*-trihydroxypropyl)flavazole.

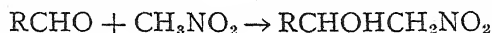
Consideration of structure III makes it evident that only two such compounds could come from the several *D*-aldohexoses. Glucose, mannose, allose, altrose, and fructose would all produce III, whereas galactose, talose, gulose, idose, and sorbose would yield the *D-threo*-isomer. All *D*-pentoses would yield the same flavazole since only one asymmetric carbon would remain, and all *L*-pentoses would give its mirror image. This suggests that the melting points of these two compounds should be identical, whereas optical rotations should be equal but of opposite sign. Ohle & Liebig found that  $212^\circ$  was the melting point for the flavazole from either *D*-xylose or *L*-arabinose, and that  $[\alpha]_D$  for the former was  $8^\circ$  and for the latter  $-8^\circ$ . Since the yields are good in the production of flavazoles, it is apparent that this is another strong tool to assist in the determination of configuration of carbohydrate molecules.

### NITRO DERIVATIVES

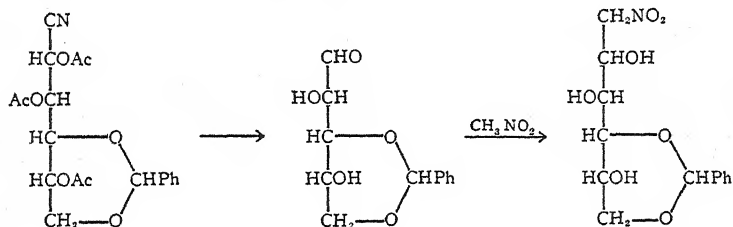
The recent commercial availability of the nitroparaffins has stimulated work with them in the carbohydrate field. Reaction of 2-nitro-1-ethanol with tetraacetylglucosyl bromide and silver carbonate causes the formation of 2-nitroethyl tetraacetyl- $\beta$ -*D*-glucoside (39). Both hydroxyl groups of 2-nitro-1,3-propanediol reacted in this fashion, whereas only one of the three in tris(hydroxymethyl)-nitromethane did so. The striking feature about these 2-nitroalkyl glucosides is the sensitivity of the glucosidic attachment towards alkaline hydrolysis.

This is in contrast to the stability of methyl, ethyl, or 4-nitrobutyl glucosides towards alkaline reagents.

Attachment of a nitro group to the carbohydrate skeleton was accomplished in a very ingenious manner by Sowden & Fischer (40). Nitromethane condenses readily with an aldehyde in the presence of a base to form a nitro alcohol:



Sowden & Fischer made use of the familiar Wohl degradation to produce the aldehyde *in statu nascendi* for this condensation. Thus, by treatment of 2,3,5-triacetyl-4,6-benzylideneglucononitrile with nitromethane and alkali there was produced 4,6-benzylidene-1-nitro-1-desoxy-D-mannitol. Acid hydrolysis of the latter converted it to 1-nitro-1-desoxymannitol. The steps may be visualized as follows:



Alkali converts this nitro compound to the *aci*-modification ( $\text{RCH}_2\text{NO}_2 \rightarrow \text{RCH}=\text{NONa}$ ) and this decomposes to mannose on contact with sulfuric acid.

#### ANHYDRO SUGARS

Methyl  $\alpha$ -D-galactoside has free hydroxyls on 2,3,4,6. By converting the 6-hydroxyl to the toluenesulfonyl derivative, and the 3,4-hydroxyls to the isopropylidene derivative, the 2-hydroxyl was then methylated ( $\text{CH}_3\text{I}$ ,  $\text{Ag}_2\text{O}$ ) with assurance (41). Removal then of the other groups by alcoholic sodium hydroxide caused production of a 3,6-anhydro ring, namely, methyl 2-methyl-3,6-anhydro- $\alpha$ -D-galactoside. In the 2,6-bis-*p*-toluenesulfonyl derivative of methyl  $\alpha$ -D-galactoside the 6-group is more readily cleaved than the 2-group, since the product formed with alcoholic sodium hydroxide is methyl 2-toluenesulfonyl-3,6-anhydro- $\alpha$ -galactoside.

Removal of the benzylidene group by acid hydrolysis of 2,4-benzylidene-1-*p*-toluenesulfonyl-5,6-anhydro-D-glucitol also involved enlarge-

ment of the anhydro ring as well as a Walden inversion in so doing, because the product obtained was 1-*p*-toluenesulfonyl-2,5-anhydro-L-*iditol* (42). The unsubstituted 6-position was proved by converting it to the 1,6-bis(toluenesulfonyl) derivative, and the *iditol* configuration was proved by the formation of an optically active dialdehyde by lead tetraacetate cleavage. The glucitol analogue would have yielded an optically inactive dialdehyde.

Anhydro compounds were also prepared by treatment of nitro derivatives with alkali (43). For example, methyl 2,3,4-triacetyl-6-nitro- $\alpha$ -D-glucoside with either aqueous or alcoholic alkali changed into methyl 3,6-anhydro- $\alpha$ -D-glucoside. Nitric esters in the 2-position behaved similarly. Such nitric esters were prepared by the action of nitric acid and phosphorus pentoxide on the appropriate methyl triacetylglucoside.

Details for the practical synthesis of levoglucosan and its triacetate from glucose pentaacetate have been presented by Coleman *et al.* (44). This, with phenol and toluenesulfonic acid, is converted to phenyl tetraacetyl- $\beta$ -D-glucoside, and the latter is broken down to levoglucosan by boiling with dilute alkali. Phenyl  $\beta$ -lactoside and phenyl  $\beta$ -cellobioside change to the corresponding sugar anhydrides (45) by similar decomposition with hot alkali. Mauthner's (46) synthesis of 3-methoxy-4-acetylphenyl tetraacetylglucoside from isopaeonol, quinoline, and tetraacetylglucosyl bromide may be mentioned here since it is an aryl glycoside. Paeonol and oracetophenone (4-acetyl-5-methyl-resorcinol) were glucosylated similarly.

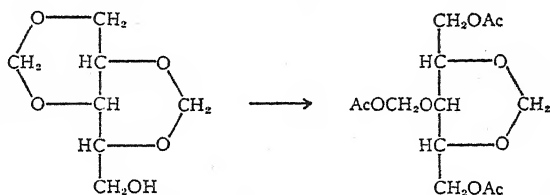
Another type of anhydride is that obtained by treatment of acetylated 2-chloroethyl glycosides with hot dilute alkali. As the acetyl groups are removed by hydrolysis, the newly created hydroxyl at position 2 undergoes cyclization with the chlorine of the chloroethyl group. 2-Chloroethyl tetraacetyl- $\beta$ -D-glucoside, its  $\alpha$ -isomer, the corresponding  $\beta$ -D-galactoside, and 2-chloroethyl heptaacetylactoside (47) have all been changed successfully in this manner. The compound formed from the glucoside may be named 1,2-ethylene- $\beta$ -D-glucose. Hydrolysis of 1,2-ethylene- $\beta$ -lactose with dilute sulfuric acid caused detachment of the galactosyl group with the formation of 1,2-ethylene- $\beta$ -D-glucose.

#### CYCLIC ACETALS

*Methylene derivatives.*—The past twelve months have seen unusual activity in this field, especially in the laboratories of W. N.

Haworth (48) and C. S. Hudson (49). Both of these investigators developed the chemistry of the methylene derivatives of various carbohydrates, substances formed by reaction of a carbohydrate at its glycol functions with formalin or trioxymethylene in the presence of either hydrochloric or sulfuric acids. The work has been concerned chiefly with derivatives of these carbohydrate materials: xylitol, adonitol, dulcitol, sorbitol, mannitol, and methyl saccharate.

Xylitol, formalin, and concentrated hydrochloric acid react to yield 1,3:2,4-dimethylenexylitol. Evidence regarding the 1,3 and 2,4 attachments for the two acetal rings was supplied by acetolysis, the reagent being a mixture of acetic anhydride, acetic acid, and a little sulfuric acid. With this treatment the 2,4-acetal was untouched, the product formed, namely, 1,5-diacetyl-3-acetoxymethyl-2,4-methylene-xylitol, having ester groups on positions 1,5 and an acylal function on position 3. The fact that deacetylation with sodium methoxide so-

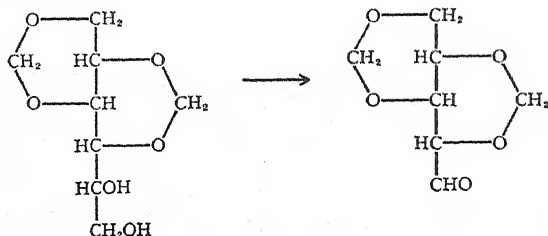


lution changed this to 2,4-methylenexylitol which was unaffected by sodium periodate, demonstrated that adjacent hydroxyls must be absent. The 2,4-blocking is the only one capable of satisfying this condition, hence the 1,3:2,4-structure for the dimethylene compound was established.

The dimethylene derivative of adonitol was made by Schulz & Tollens in 1896, but its structure as D,L-1,3:2,4-dimethyleneadonitol was not proved till 1944 by the work of Hann & Hudson. The proof was the same as that developed for dimethylenexylitol. Here again, acetolysis and saponification yielded 2,4-methyleneadonitol, showing greater tendency for acetal cleavage at the primary position than at the secondary.

Formaldehyde converts dulcitol to 1,3:4,6-dimethylenedulcitol. Sorbitol yields not only dimethylenesorbitol, but also a trimethylene derivative if the reaction time is extended to four days at 50°. That 1,3:2,4-dimethylene-D-glucitol is the structure of the former was substantiated by oxidation to 2,4:3,5-dimethylene-L-xylonaldehyde by

sodium periodate. The trimethylene derivative was the 1,3:2,4:5,6-trimethylene-D-glucitol. The 2,4-attachment was the only one resisting acetolysis, again demonstrating the greater reactivity at the two



primary positions. Proof that 2,4-methylene-D-glucitol was the resultant compound rested in the fact that only two of the four hydroxyl groups were adjacent to each other as shown by the oxidation with lead tetraacetate.

With one of the terminal positions of sorbitol blocked as in the 1,3:2,4-dimethylene derivative it becomes possible to place a *p*-toluenesulfonyl group at the other terminal position. Ness, Hann & Hudson prepared this compound and converted it to the 6-iodo derivative. The latter was reduced to 1,3:2,4-dimethylene-6-desoxy-D-glucitol by hydrogen under slight pressure with Raney nickel catalyst. This is the dimethylene derivative of D-epirhamnitol.

It is interesting to point out that whereas toluenesulfonic esters of carbohydrates and other aliphatic esters react with sodium iodide in acetone at 100° to form organic iodides, the toluenesulfonic ester of phenol is without effect on sodium iodide (50).

Starting with 1,6-dibenzoylsorbitol Haworth & Wiggins were able to prepare a dimethylenesorbitol which was isomeric with the one mentioned above. Obviously the methylene groups were on the 2,3,4,5 positions, but Hann, Wolfe & Hudson proved that the acetals were also of the 6-membered cyclic variety with attachments at 2,4 and 3,5. Proof was by acetolysis and saponification as before of the acylal-ester to the known 2,4-methylene-D-glucitol. Various 1,6-derivatives of the 2,4:3,5-dimethylene-D-glucitol were prepared (48), such as the dichloride, the bistriphenylmethyl ether, the bis-*p*-toluenesulfonic ester. Also, with chromic acid in acetic acid and subsequent esterification it was converted to methyl dimethylenesaccharate, which compound was also prepared directly from potassium hydrogen saccharate, trioxymethylene, and sulfuric acid with subsequent esterification.

One noteworthy feature concerning this methyl 2,4:3,5-dimethylenesaccharate is its ability to epimerize to the extent of 33 per cent to dimethylene-L-idosaccharic acid during six hours of refluxing with barium hydroxide.

Haworth, Heath & Wiggins (48) applied the Gabriel synthesis to 1,6-dichloro-2,3,4,5-dimethylene-1,6-didesoxy-D-mannitol by heating it at 200° with potassium phthalimide. The 1,6-diphthalimido derivative thus obtained was converted to the 1,6-diamine by treatment with alcoholic hydrazine. This diamine (1,6-diamino-2,3,4,5-dimethylene-1,6-didesoxy-D-mannitol) was converted to a resin by heating with adipic acid for three hours at 210°. Fibers could be pulled from the mass as it cooled but, differing from nylon, these fibers could not be cold-drawn.

*Benzylidene derivatives.*—By refluxing in a mixture of 80 per cent alcohol and acetic acid 1,3:2,4-dibenzylidene-D-glucitol is converted into 2,4-benzylidenegluclitol (51). Here also, the acetal at the primary position is the one more readily broken.

Reaction of methyl  $\alpha$ -D-glucoside with benzaldehyde yields methyl 4,6-benzylidene- $\alpha$ -D-glucoside, from which the 2,3-bisphenylcarbamyld derivative was then obtained by reaction with phenyl isocyanate (52).

1,3-Benzylidene-D-arabitol, m.p. 152°, was prepared by passing a rapid stream of hydrogen chloride gas into a mixture of D-arabitol and benzaldehyde (53). The isomeric 2,3-benzylidene-D-arabitol, m.p. 81°, was made from 1,5-dibenzoyl-D-arabitol, benzaldehyde, and zinc chloride, followed by saponification. Proof of the 2,3-attachment rested on the formation of a D-threose derivative through periodate oxidation, whereas an erythrose derivative would have been formed from a 3,4-attachment.

Extension of a previously developed method was successful for the synthesis of 2-caproylglycerol and 2-caprylylglycerol (54). In this synthesis 1,3-benzylideneglycerol, caproyl chloride or caprylyl chloride, and pyridine were mixed, after which the benzylidene group was removed by hydrogenation in the presence of palladium.

*Ethylidene and isopropylidene derivatives.*—Glucose and paraldehyde react in the presence of a trace of sulfuric acid to form a mixture of monoethylideneglucoses (55). Mannose, galactose, and fructose behave similarly. Maltose and sucrose yield diethylidene derivatives. In this manner also, methyl 3-methylglucoside is converted into methyl 3-methyl-4,6-ethylideneglucoside (56).

An isopropylidene derivative was used in the synthesis of 4-meth-

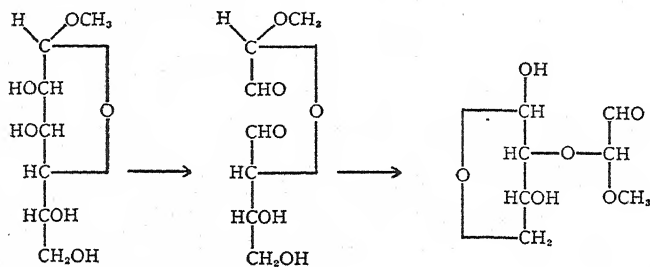


yl-D-mannonic acid (57). Potassium 2,3:5,6-diisopropylidene-D-mannonate was first prepared and methylated by methyl sulfate and alkali, then hydrolyzed to the desired compound. It was then converted to the lactone, amide, and phenylhydrazide for polarimetric studies. Both the amide and the hydrazide were found to be less dextrorotatory than the acid itself, which is in agreement with the amide and phenylhydrazide rules of rotation, the L-grouping, characteristic of the  $\alpha$ -hydroxy group, being the determining factor.

#### THE USE OF LEAD TETRAACETATE IN DETERMINING CONFIGURATIONS

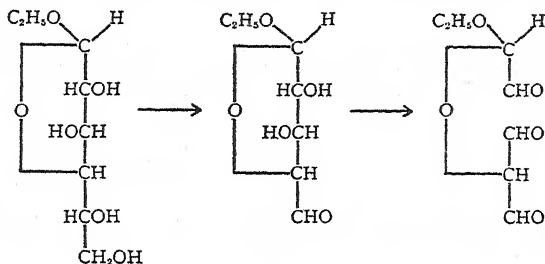
It has been known for some time that lead tetraacetate cuts glycolic carbon-to-carbon bonds, and Hockett has shown that in cyclic systems *cis* 1,2-diols are split more rapidly than *trans* 1,2-diols. In open chain compounds, however, such as erythritol, mannitol, etc., no such difference in rate is discernible among isomers. The lead tetraacetate oxidation was used to confirm previously established structures of 1,6-dibenzoyl-D-mannitol, 1,2,6-tribenzoyl-D-mannitol, 1,6-dibenzoyl-D-glucitol, as well as the new compound 1,2,6-tribenzoyl-D-glucitol (58, 59). The tribenzoyl compounds, for example, yielded dibenzoyl-glyceraldehyde.

In agreement with prediction methyl  $\alpha$ -D-mannofuranoside, because of its *cis* hydroxyls, rapidly consumed one mole of lead tetraacetate with no more than a trace of formaldehyde production (60). It was reasoned that the rapid formation of aldehyde groups at the 2,3-positions would prevent continued reaction at 5,6 because of hemiacetal formation between the 6-hydroxyl and an aldehyde group thereby removing the glycol configuration at 5,6 which (open chain) would otherwise react slowly with the oxidant.



Prediction and experiment coincided in the work with ethyl  $\beta$ -D-galactofuranoside which is of the *trans* glycol type at 2,3. Hence, lead

tetraacetate reacts first at the 5,6-positions with the production of a mole of formaldehyde. The unbroken furanose ring prevents cyclization with the new aldehyde group on atom 5, so a second mole of lead tetraacetate is consumed gradually during scission at positions 2,3.



This method is of utility, therefore, in determining the configurations on atoms 2,3.

#### ANALYSES BY FERMENTATION METHODS

D-Galactose may be analyzed with an accuracy of 92 to 98 per cent by selective fermentation with the yeast *Saccharomyces carlsbergensis*, as contrasted to the nonfermentation with *S. bayanus*. Both of these yeasts ferment glucose, mannose, and fructose, but do not ferment pentoses or glucuronic acid (61).

Gottschalk (62) has suggested a method for determining the furanose-pyranose ratio in D-fructose solution at equilibrium at 0°. Ordinary fructose (the pyranose form) is hardly affected by bakers' yeast in 0.1 M potassium dihydrogen phosphate solution at 0°; in contrast, glucose ferments readily, the presumption being that the hydroxyl on carbon 6 must be free if the compound is fermentable. The furanose form of fructose does have the 6-hydroxyl free and it is this form of fructose which is fermentable. Using this as an analytical method Gottschalk determined that at 0° an equilibrated solution of fructose in 0.1 M potassium dihydrogen phosphate contained 12 per cent of the furanose form. It is estimated that at 20°, one fifth of it is in the furanose modification.

#### ASCORBIC ACID

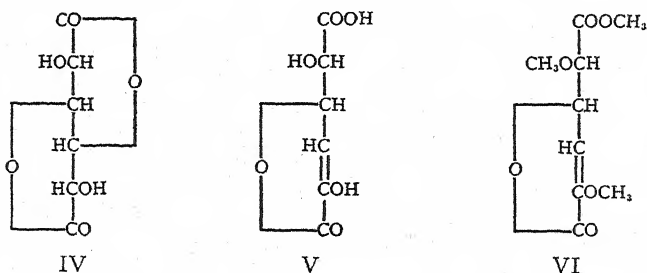
The 2,3-diacetate of ascorbic acid was made by passing ketene into ascorbic acid. This hydrolyzes rapidly at 32°, but slowly at 0°. 3-Acetyl-5,6-isopropylideneascorbic acid was prepared from 5,6-isopropylideneascorbic acid and ketene (63). Dehydroascorbic acid undergoes an interesting quantitative hydrolytic cleavage into oxalic acid at

pH 7 in the presence of phosphate ion (64). Presumably threose is the other product. No oxalic acid was formed unless phosphate ion or cyanide ion was present.

A new process for the preparation of ascorbic acid from such pectic substances as beet pulp has been announced by Isbell (65). First an enzymatic hydrolysis to sodium calcium galacturonate takes place, then on hydrogenation (Raney nickel catalyst) the L-galactonate is formed from which 1,4-L-galactonolactone is readily obtained. Oxidation of this lactone to 2-oxo-L-galactonic acid is accomplished in 25 to 30 per cent yields by sodium chlorate in the presence of vanadium pentoxide. (Yields in other steps were over 90 per cent.) The final steps are esterification of the lactone to methyl 2-oxo-L-galactonate by methanol and hydrogen chloride, then lactonization and enolization of the oxo ester by sodium methoxide.

The sodium methoxide method of lactonization and enolization of methyl 2-oxo-D-gluconate gives rise to D-arabo-ascorbic acid (66). Ozonolysis of its 2,3,5-trimethyl derivative gives rise to oxalic acid and 3-methyl-D-erythrionolactone.

D-manno-Saccharodilactone differs from most lactones in the sugar series in that it reduces Fehling solution. Kiliani made this observation in 1887. An explanation comes as a result of recent work by Haworth *et al.* (67). With methyl iodide and silver oxide the dilactone (IV) changed to methyl 3,6-lactono-D-manno-4-desoxy-2,5-dimethoxy-4-hexenedioate (VI), as if it were preceded by isomerization of IV to V under the influence of alkali. Methylation of the



hydroxyls deprives VI of its reactivity towards Fehling solution, but the compound still adds bromine. Ozone converts it to oxalic acid and 2-methoxy-3-hydroxy-L-erythro-succinaldehydic acid. Structure V resembles the ascorbic acids in that it is an unsaturated lactone of carbohydrate character but differs in having only one hydroxyl at the

olefin function. Also it differs in possessing a carboxyl group. It may be assumed that the enol position of structure V is involved in Rehorst's iodometric procedure ( $I_2 + NaOH$ ) for the analysis of *manno*-saccharic dilactone (68). In this procedure, iodoform is obtained.

#### PROPARGYL AND ALLYL DERIVATIVES

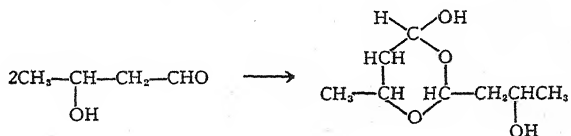
Propargyl  $\beta$ -D-glucoside is an interesting material prepared by the action of potassium ethoxide on 2-bromoallyl  $\beta$ -D-glucoside (69). When mixed with glucose and treated with Fehling solution it prevents the precipitation of cuprous oxide by converting the latter to a soluble cuprous salt.

Methyl tetraallyl- $\alpha$ -D-glucoside was prepared from allyl bromide and methyl  $\alpha$ -D-glucoside (70). Interest in this compound comes from its tendency to polymerize to a colorless transparent resin under the influence of oxygen and heat. Benzoyl peroxide seemed to be ineffective as a polymerization catalyst.

#### ALDOL AND ITS POLYMERS

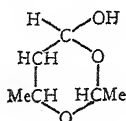
Aldol and aldoses have a feature in common in that both may be regarded as  $\beta$ -hydroxy aldehydes. Spaeth (71) and Murray (72) have played prominent parts in recent developments of the chemistry of aldol polymers, but others (73) have participated.

The acetate of monomeric aldol,  $CH_3CHOAcCH_2CHO$ , was prepared for the first time by Spaeth in 1943 by maintaining a mixture of aldol, acetic anhydride and toluene at sufficiently high temperature ( $115^\circ$ ) to inhibit dimerization. Aldol reacts with itself with great readiness to form a cyclic hemiacetal:

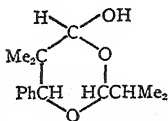


Other aldols dimerize similarly, but the chemistry is complicated if other aldehydes are present. Acetaldehyde, for example, is usually present with aldol and the two combine in a process resembling polymerization to form 2,4-dimethyl-6-hydroxy-1,3-dioxane (VII). In the presence of potassium carbonate benzaldehyde and isobutyraldehyde form an aldol,  $\text{Ph}-\text{CHOH}-\text{CMe}_2-\text{CHO}$  (VIII), but this reacts further with isobutyraldehyde to produce the cyclic hemiacetal (IX). It is interesting to note that IX decomposes into VIII at  $150^\circ$ .

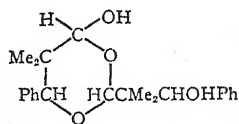
(12 mm.) and that IX yields the phenylhydrazone of VIII on reaction with phenylhydrazine. On standing, VIII dimerizes to a crystalline



VII



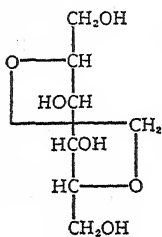
IX



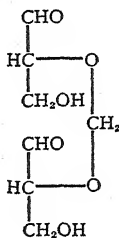
X

product (X). Isobutyraldehyde itself polymerizes in similar fashion in the presence of dibutylamine to form 2,4-diisopropyl-5,5-dimethyl-6-hydroxy-1,3-dioxane (structure like IX).

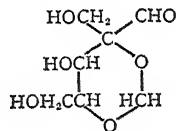
Reactions involving aldolizations were encountered by Hudson *et al.* (74) after oxidizing 2,5-methylene-D-mannitol (XI) with sodium periodate and treating the methylenebis(2-D-glycerose) (XII) thus obtained with barium hydroxide solution. An intramolecular aldol condensation occurred to produce the cyclic acetal (XIII). Since



XI



XII



XIII

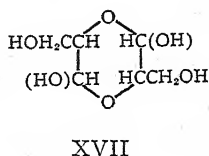
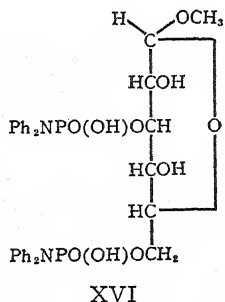
XIII, like other aldols, is a  $\beta$ -hydroxy aldehyde it should be capable of reacting with aldehydes, including itself, in the manner outlined above.

The methylene derivative (XI) is sufficiently novel to deserve comment for it carries the unusual 7-atom ring. Its structure seems established because of its oxidation to XII without loss of carbon. It was prepared by acetolysis of the trimethylene-D-mannitol of Schulz & Tollens (1896). This yielded a diacetyl-bisacetoxymethyl-methylene-D-mannitol which underwent cleavage with sodium methoxide to form the 2,5-methylenemannitol.

#### PHOSPHATES AND SULFATES

Protected acid chlorides of phosphoric acid such as  $(C_6H_5)_2-NPOCl_2$  (XIV) or  $(C_6H_5O)_2POCl$  (XV) have been found to be

useful reagents with which to introduce phosphate functions into carbohydrate molecules. Hydroxyls at positions 3 and 6 of methyl  $\alpha$ -D-glucoside are attacked by the acid chloride (XIV) to form the glucoside phosphate of structure XVI (75).



Glycerose is both an  $\alpha$ - and  $\beta$ -hydroxy aldehyde. Its dimerization could be explained, therefore, in the manner of aldol dimers (cf. structures VII to X), but the structure given to glycerose dimer (XVII) involves the  $\alpha$ -hydroxyls. This structure possesses two hydroxyls of primary alcohol character and two (in parentheses) which are aldehydic in nature. With reagent XV all four hydroxyls are replaced by  $\text{OPO}(\text{OC}_6\text{H}_5)_2$  groups (76). If this tetrphosphate is treated with hydrobromic and acetic acids only the two of these groups which are on the aldehydic positions are replaced by bromine. Hydrogenation of the dibromide with a platinum catalyst causes reduction of the remaining two  $\text{OPO}(\text{OC}_6\text{H}_5)_2$  groups to  $\text{OPO}(\text{OH})_2$  groups. After purification by means of its crystalline dioxane addition complex, water brings about a rapid depolymerization of the last compound to glyceraldehyde 3-(dihydrogen orthophosphate),  $(\text{HO})_2\text{PO}-\text{O}-\text{CH}_2\text{CHOHCHO}$ .

Sulfuric esters of cellulose and carboxymethylcellulose have been tested as substitutes for heparin as an anticoagulant for blood (77). The esters were prepared by the action of chlorosulfonic acid on the carbohydrate material in the presence of pyridine. Cellulose trisulfate was found to be extremely toxic, although only one fourth to one sixth as effective as heparin. Carboxymethylcellulose sulfate was one tenth as toxic as cellulose trisulfate and possessed one fifth the effectiveness of heparin, the effect, however, lasting even longer than that of heparin. The necessity for taking a carbohydrate of very large molecular weight was demonstrated by studying levoglucosan sulfate which displayed no anticoagulant properties whatsoever.

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## THE CHEMISTRY OF THE LIPIDS

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In the present review, attention has necessarily been confined to those publications dealing with the chemistry of the fatty acids, glycerides, and phospholipids and to the use of physical methods in studying their properties. Although earlier reviews have not included a treatment of fat oxidation and antioxidants, the current importance and widespread interest in these subjects makes it advisable to give them brief consideration. No mention of the many fatty acid analyses of various seed fats has been included. The reader will find the latter material well summarized each year in Piskur's review (1, 2). Also excluded from consideration here are the many technological applications of fats and their derivatives.

### METHODS

A report of the Committee on Analysis of Commercial Fats and Oils (3) on cooperative tests carried out in six different laboratories on identical samples of cottonseed, soybean, peanut, and corn oils showed very poor agreement in the calculation of fatty acid and triglyceride composition from iodine and thiocyanogen values. The poor agreement observed prevents any conclusions at this time on the justification of the basic assumption underlying these calculations that there is a stoichiometric addition of thiocyanogen to acids and glycerides.

Spectrophotometric methods for the quantitative analysis of polyene acids, such as linoleic, linolenic, and arachidonic, have now been perfected to the point where the older and less reliable thiocyanogen determinations seem bound to be replaced to a considerable extent, at least in those laboratories that possess the necessary physical equipment. In its annual progress report to the American Oil Chemists' Society, the Committee on Analysis of Commercial Fats and Oils (4) called special attention to the desirability of adopting the spectrophotometric method at least as an alternate for the thiocyanogen value. The methods may be adapted for the measurement of both conjugated and nonconjugated diene, triene, and tetraene acids present in a specimen.

A necessary prerequisite for the use of spectrophotometry in fat analysis is the assurance of satisfactory reference materials. It would be most desirable to have pure specimens of unsaturated acids whose steric configurations were known with certainty. Some progress has been made in this direction by the use of acids purified through their crystalline bromides. Linoleic and linolenic acid standards have been prepared by this means and reported on before (5), and this year Beadle & Kraybill (6) have confirmed the earlier report on the specific absorption coefficients of the alkali isomerized soaps of these acids and, in addition, have given similar data for a sample of arachidonic acid which was probably 95 per cent pure. The reference values for these three acids may need to be altered slightly as additional information accumulates from several laboratories on highly purified acids prepared by different techniques. Irrespective of this fact, the data listed below (6) will prove invaluable in fat studies involving measurements of highly unsaturated acids:

Isomerized Fatty Acid Soap Using Ethanol as a Solvent	Specific Absorption Coefficients at Various Wave Lengths			
	At 2340 Å	At 2680 Å	At 3010 Å	At 3160 Å
Arachidonic .....	59.3	53.4	25.8	22.6
Linolenic .....	60.9	53.2	....	....
Linoleic .....	86.0	....	....	....

Barnes *et al.* (7) measured, at 2100 to 2500 Å, the spectral absorption of the same three unsaturated acids mentioned above and of oleic and stearic acids also. It was shown that the extinction coefficients determined at 2100 Å for coconut, olive, and corn oils agreed reasonably well with the extinction coefficients calculated from the fatty acid analyses. Raman spectroscopy of a group of saturated fatty acids was reported by Hende & Fonteyne (8).

A paper on spectral studies by Brode *et al.* (9) provides a method for determining the amount of two, three, and four double bond conjugated acids in the presence of nonconjugated unsaturated acids. A series of simultaneous equations were set up as follows:

$$\begin{aligned}
 1.0 y + 2000 z &= 100 A = 100 E_{1\text{ cm.}}^{1\%} \text{ at } 938 f. \\
 0.1 x + 1320 y + 700 z &= 100 B = 100 E_{1\text{ cm.}}^{1\%} \text{ at } 1071 f. \\
 1200 x + 112 y + 60 z &= 100 C = 100 E_{1\text{ cm.}}^{1\%} \text{ at } 1290 f.
 \end{aligned}$$

where  $A$ ,  $B$ , and  $C$  represent  $E_{1\text{ cm.}}^{1\%}$  observed at the respective frequencies ( $f$ ), and  $x$ ,  $y$ , and  $z$  represent the percentage of two, three,

and four conjugated double bonds, respectively. The numbers are the  $E_{1\text{cm.}}^{1\%}$  values of the respective components at a particular frequency. On solution of the equation, and cancellation of very small terms, the equations become

$$x = 0.0833 C - 0.00706 B$$

$$y = 0.0758 B - 0.0269 A$$

$$z = 0.0500 A$$

Thus a measurement of  $E_{1\text{cm.}}^{1\%}$  at the three frequencies indicated, plus a simple calculation, provides the desired percentages of conjugated diene, triene, and tetraene acids.

Although Brode *et al.* (9) reported that purified linoleic acid specimens prepared by crystallization techniques yielded a product more nearly free from conjugation than they could obtain by debromination procedures, Wood *et al.* (10) prepared linoleic acid by debromination of tetrabromostearic acid in ether solution with very low preformed conjugations.

Norris & Buswell (11) found no basis for the precaution frequently taken against using Wijs solution more than thirty days old for iodine value determinations. They found that the solution could be stored for more than a year in dark colored bottles at room temperature. Rowe, Furner & Bliss (12) propose the use of pyridine sulfate dibromide in conjunction with mercuric acetate as a bromine addition reagent for determining the iodine value of highly unsaturated and conjugated systems such as resin acids, tall oil, tung oil, linseed oil, natural resins, synthetic rubberlike materials, etc. MacLachlan (13) points out that iodine value determinations of phospholipids can be very erratic when chloroform is used as a solvent for material precipitated with acetone and magnesium chloride. To obtain reproducible results it was necessary to evaporate the chloroform solution to dryness.

A method for the estimation of mono-unsaturated and di-unsaturated glycerides has been proposed by Sukumaran & Menon (14). It is based on (a) the equivalence of the ratio of saturated acids in the original glycerides to those in the mono- and diazelaic glycerides formed during acetone permanganate oxidation of the whole fat, and (b) the nonextractability of the monoazelaic glycerides from ether solution by aqueous bicarbonate solution. The amounts of individual saturated acids being known, the theoretical saponification values of the mono- and diazelains can be calculated, and from these values and

the two experimental values, the mono- and diazelains, and thus the mono- and di-unsaturated glycerides in the original sample can be determined.

The study by Krober & Collins (15) indicates clearly how great an effect humidity has on the accurate determination of the total lipid in soybeans. Unless moisture conditions are carefully controlled, the determination is empirical.

The fairly recent isolation of higher fatty aldehydes from animal tissues (16) makes it desirable to have methods for their quantitative determination. Anchel & Waelsch (17) have made use of the color developed by aldehydes with fuchsin sulfite for this purpose. They used synthetic palmitaldehyde and stearylaldehyde glyceryl acetals as standards to study the behavior of the fuchsin reagent with palmitaldehyde, stearylaldehyde, lower aldehydes, and several aldehyde derivatives. The synthetic acetals did not react exactly like the aldehyde complexes from natural sources. Although this fact may cast doubt on glycerol acetal as the naturally occurring form of the aldehyde, Anchel & Waelsch believe the synthetic products may be used as a standard for the determination of the naturally occurring compounds.

Boyd (18) emphasizes again that either plasma or serum should be selected for lipid analyses rather than whole bloods especially when "only one analysis or one set of analyses is to be done"; and Teeri (19) offers a further modification in the determination of esterified cholesterol.

The microestimation of total volatile fatty acids in blood is described by McClendon (20); only 1 ml. of blood is required. Mitchell, Smith & Money (21) have developed a semimicroprocedure, readily adaptable to routine analyses, for the saponification of esters. Lykken *et al.* (22) describe a potentiometric determination of acidity in highly colored materials which should find broad applications in the measurement of fatty acid and saponification numbers. Fehnel & Amstutz (23) propose the use of ethyl-2,4-dinitrophenyl acetate as an indicator in the determination of saponification numbers in dark-colored oils.

#### FATTY ACIDS

*Unsaturated acids.*—The probable occurrence of small amounts of an octadecenoic acid other than oleic acid in lard, beef tallow, beef adrenal phospholipids, pork liver lipids, human adipose tissue, and soybean and rapeseed oils is indicated by Millican & Brown (24). Their evidence rests mainly on the fact that the melting points of oleic acid

specimens, purified by a combination of fractional distillation and fractional crystallization of the methyl esters, and the melting points of their dihydroxy derivatives, were lower by one to three degrees in the case of specimens derived from the fats mentioned above than of specimens derived from olive or cottonseed oils. In the case of lard, about 1 per cent of the  $C_{18}$  esters was eventually isolated as an octadecenoic acid, iodine value 88.6, two samples of which melted at 0.5 to 1.3°, and a third at 2.5 to 3.3°; in contrast, oleic acid from olive oil had an essentially theoretical iodine value, 90.0, and a melting point of 13.3 to 13.5°. Further studies of these unusual octadecenoic acids are needed to determine their exact structure.

Octadecadienoic acids not strictly comparable with the ordinary seed fat linoleic acid have been reported from time to time. Again this year, Baldwin & Longenecker (25) observed an acid in human milk fat; de la Mare & Shorland (26) reported a similar finding in the case of sow milk fat; and Shorland (27) implies the presence of such an acid in the leaf lipids of forage grasses and clovers. In each case it was impossible to obtain the characteristic crystalline tetrabromostearic acid derivative, although bromine analysis of the liquid bromides prepared from  $C_{18}$  ester fractions (saponification equivalent, 294.7) showed them to be mainly tetrabromostearic acid. Baldwin & Longenecker presented additional verification for the presence of appreciable amounts of octadecadienoic acid in human milk fat. Alkali-isomerized soaps of the  $C_{18}$  fractions showed the typical conjugated diene absorption peak at 234 m $\mu$ . Although Hilditch & Meara (28) were able to isolate from human milk fat a crystalline tetrabromostearic acid with a melting point of 110 to 112°, they infer the presence of an octadecadienoic acid other than linoleic acid (cis-cis- $\Delta^{9,12}$ -octadecadienoic acid).

At various times the suggestion has been made that certain oils, particularly those from fish livers, contain trienoic acids other than the  $C_{18}$ , linolenic, acid. Usually, however, there has not been a convincing recognition of these materials. Baudart (29) has now isolated and determined the structures of the  $C_{20}$  and  $C_{22}$  trienoic acids of *Carcharodon carcharias* oil. Fractional distillation for preliminary concentration of the desired acids, followed by fractional crystallization of their sodium salts, yielded materials which, in terms of the analytical constants, were not pure but highly concentrated: for the  $C_{20}$  trienoate, saponification number 176 (theory 175), iodine value 252 (theory 238); and for the  $C_{22}$  trienoate, saponification number 162

(theory 161), and iodine number 240 (theory 219). The products of ozonolysis led to the following postulated structures,  $\Delta^{8,11,14}$ -eicosatrienoic acid and  $\Delta^{8,11,14}$ -docosatrenoic acid. Thus these trienoic acids are comparable with the series of dienoic acids from the same oil reported earlier to be  $\Delta^{11,14}$ -acids, and both the trienoic and dienoic acids of this oil differ from the customary linoleic and linolenic acids which are  $\Delta^{9,12}$  and  $\Delta^{9,12,15}$  acids, respectively.

One of the tetraenoic acids, the  $C_{22}$ , of the same oil appears also to have an analogous structure (30), although the position of the two unsaturated linkages furthest removed from the carboxyl could not be fixed with certainty. The products of ozonolysis made it impossible to decide among the following structures,  $\Delta^{8,12,16,20}$ -docosatetraenoic acid,  $\Delta^{8,12,16,19}$ -docosatetraenoic acid, and  $\Delta^{8,12,15,19}$ -docosatetraenoic acid.

*Branched chain acids.*—The appearance of branched chain fatty acids has been restricted to a very few natural fats. It is, therefore, of considerable interest that Weitkamp (31) has found a series of them in degreas, which is predominantly wool fat.

Additional evidence for the proposed structure of naturally occurring tuberculostearic acid as 10-methyl stearic acid, was presented by Velick (32). X-ray diffraction studies of the amides of natural tuberculostearic acid and of synthetic *dl*-10-methylstearamide revealed differences, particularly in the higher orders of the intensity distributions of the 00 $l$  reflections of the two compounds, which are consistent with the earlier suggestion that the natural acid is a single optical isomer although it shows no observable optical activity. In order to explain the difference of 10° in the melting points of the natural and the synthetic acids, it has been alternately considered that the natural product might include a small amount of a position-isomer (e.g., 9-methylstearic acid). This latter suggestion seems now definitely to be ruled out by Velick's evidence.

A new fatty acid for which the name phytomonic acid is proposed by Velick (33) has been isolated from the acetone-soluble fat of the crown-gall bacillus, *Phytomonas tumefaciens* (34, 35). Velick & Anderson (35) isolated the acid from the hydrogenated liquid acids by taking advantage of the greater solubility of its lead salt in ether. The acid was declared to be a liquid saturated fatty acid, of the probable empirical composition  $C_{20}H_{40}O_2$ . Since the separation of the acid from a group of unsaturated acids required their hydrogenation it was not clear that the acid itself was saturated. Velick (33) showed,

however, that the ratio of unsaturated acids in the original mixture to the normal saturated acids in the reduced mixture was approximately unity and on this basis he concluded that the acid occurs in the bacterial cell in the saturated form. The physical properties of the acid, e.g., a melting point more than  $50^{\circ}$  lower than corresponding straight chain compound and the solubility of its lead salt in ether, are strongly suggestive of a branched chain structure. X-ray diffraction methods were used (36) to establish the provisional structure as 10- or 11-methylnonadecanoic acid.

In continuation of their program for the synthesis of branched chain acids, Cason & Prout (37) prepared keto ester derivatives in the  $C_{19}$  and  $C_{25}$  series, and Cason *et al.* (38) developed a satisfactory general method for synthesizing branched chain esters.

*Biological aspects.*—Although the component acids of cow milk fat have frequently received detailed consideration, little comparable study has been given to human milk fat. A variety of factors combined last year, however, to yield two independent investigations of human milk fat by Hilditch & Meara (28) and by Baldwin & Longenecker (25).

It is interesting to consider the results of these two studies together with comparable data for sow's milk fat (26), cow colostrum fat (39), and cow milk fat (40). It is immediately apparent that a much smaller proportion of low molecular weight acids are found in human and sow milk fat than in cow milk fat. Whereas cow milk fat had 22 to 26 per cent acids in the  $C_4$  to  $C_{12}$  group, mature human milk had only 8 to 12 per cent and the sow milk fat had an almost negligible proportion—2.4 per cent. Hilditch & Meara concluded that no acids of lower molecular weight than capric acid are present in human milk fats, although Baldwin & Longenecker reported the presence of traces of these acids. Both the human and the sow milk fats had larger proportions of  $C_{18}$  diene acids and  $C_{20-22}$  acids than are customarily found in cow's milk.

To a considerable extent, biological investigations on the relative nutritive value of fats for growth, lactation, prevention of abnormal skin conditions, etc., are dependent upon the satisfactory clarification of the structure of the minor component fatty acids. Thus it has been demonstrated (41, 42) that inclusion of butter fat or the fatty acids from butter fat in a rat's ration provides better growth than most vegetable oils when the carbohydrate of the ration is lactose. Boutwell *et al.* (43) showed that this was not due to a greater food consumption



because of the preference for butter flavor, the substance diacetyl. It will be recalled that Schantz, Elvehjem & Hart (44) first postulated the existence of a component of butter fat with special growth-promoting properties which was later ascribed to the saturated non-volatile acids of butter fat (45, 46). The active factor could also be produced by hydrogenating the unsaturated acids of butter fat (47). To date, however, there have been no reports of unusual acids in butter fat not previously described. Oils containing the same acids which are known to occur in butter fat have not been observed to have the same biological effect.

In contrast with the results of the Wisconsin studies are the results of Deuel and his colleagues (48 to 51). In the first paper of the series (48), data are presented which fail to substantiate the suggestion that butter fat contains unusual saturated acids essential for growth and not present in other fats. (Diets used were made up of 70.6 per cent mineralized skim milk and 29.4 per cent of various fats—butterfat, margarine fat, corn oil, cottonseed oil, olive oil, peanut oil, and soybean oil.) The results of Zialcita & Mitchell (52) are essentially in agreement with these findings.

One means frequently employed in nutritional investigations to determine the adequacy of a diet with respect to individual components is to use the diet under conditions in which the requirement of the particular component is most stringent and specific. Animal needs during reproduction and lactation have been resorted to in this connection. Deuel *et al.* (51) observed no differences in the fertility of the male or female rats used in the above mentioned experiment; and, on the basis of the animal weight at fourteen or twenty-one days, the diets were equally effective in promoting lactation, irrespective of the fat present in the diet.

Maynard's earlier studies had shown that dietary fat stimulates milk secretion in dairy cows. In a recent experiment (53) an attempt was made to investigate the relation of dietary fat to lactation in rats by measuring the growth of litters whose mothers were fed a diet free from fat as compared with a diet containing varying amounts of corn oil and hydrogenated fat. The litter performance was satisfactory when corn or coconut oil was included, but was inferior on a low-fat diet or when hydrogenated corn oil (iodine value 0.6), hydrogenated coconut oil, or ethyl linoleate was included. It seems clear from these studies that certain unsaturated acids stimulate lactation but that the effect is not due to linoleic acid.

Another example of a special dietary study, in which under a particular stress an effect of fatty acids is observed, is found in the reports of Dam that the symptoms of vitamin E deficiency in chickens can be accelerated by the inclusion in the diet of highly unsaturated fatty acids (54) and that chickens reared on a diet deficient in vitamin E developed severe encephalomalacia when the diet contained about 4 per cent of highly unsaturated fatty acids of hog liver (55). Both exudative diathesis and encephalomalacia were observed when these acids were fed in smaller amounts corresponding to about 0.5 per cent of the diet. The acids used in the latter experiment were prepared by low temperature fractional crystallization of the mixed acids from hog liver; most effective was the fraction remaining in acetone solution at  $-72^{\circ}$ , iodine value 242. In view of the facts (*a*) that the two symptoms, exudative diathesis and encephalomalacia, are dependent upon the ingestion of highly unsaturated fatty acids (palmitic, stearic, and oleic have no effect), (*b*) that the affected tissues (adipose and brain tissues) are rich in lipids, and (*c*) that the first changes are fine hemorrhages followed by edema or exudation, Dam suggests that the same basic biochemical disturbance may be occurring in different tissues. In view of the work of Fitzhugh, Nelson & Calvery (56), in which pathological lesions similar to those occurring in vitamin E deficiency were observed in rats fed experimental diets containing rancid lard, it should be pointed out that Dam's results were apparently not due to rancid fat samples.

Because of the practical importance of hydroxy acids as surface active agents in food products, the publication by Harris *et al.* (57) is of interest. A synthetic glyceride containing all of the acids as dihydroxystearic acid was fed to weanling rats at 2.2 per cent and 2.5 per cent levels in replacement of equal weights of hydrogenated fats in control diets. The hydroxy acids appeared to exert a favorable effect on the growth and development of rats on dietaries presumed to be adequate.

Beveridge (58) has confirmed Engel's earlier observations (59) that the lipotropic action of choline is increased by the presence of corn oil in the diet. On the other hand, the lipotropic effect of inositol is obliterated by the inclusion of corn oil.

Miller *et al.* (60) have made the interesting observation that the incidence of hepatic tumors in rats fed *p*-dimethyl-aminoazobenzene is high when corn oil is used in the diet but exceedingly low when hydrogenated coconut oil is used. A number of possible explanations were

considered but no conclusion could be drawn from the available evidence.

Kodicek & Worden (61) have confirmed the suggestions that the growth of microorganisms may be considerably affected by the presence of fatty acids in the medium. Oleic acid, and more especially linoleic and linolenic acids, suppressed the growth of *Lactobacillus helveticus*, a test organism for microbiological riboflavin assays. Other Gram-positive organisms were similarly affected. Also, linoleic acid appeared to be highly toxic when injected parenterally into mice.

*Physical behavior.*—Interest in the physical behavior of fatty acids and their derivatives has manifested itself in several publications.

Schuette *et al.* (62) have continued their study on solidification point curves of binary acid mixtures by presenting solidification point diagrams of binary mixtures of tetracosanoic and triacontanoic acids. Grondal & Rogers (63) have demonstrated that the purity of low molecular weight fatty acids,  $C_6$  to  $C_{12}$ , can be determined readily to an accuracy of 0.25 per cent present in a concentration of 80 per cent or more by means of the melting points of binary mixtures.

Efforts during the past year have been made also to determine more precise physical constants for both fatty acids and their methyl esters. Althouse & Triebold (64) have shown that, with the exception of the  $C_{18}$  methyl esters, an ester fraction of  $C_6$  to  $C_{18}$  can be identified and its purity ascertained by means of its vapor pressure curve. Their data, which included decomposition temperatures and pressures, make possible the elimination of excess decomposition during ester distillation. The solubilities of the fatty acids from caprylic to stearic acids inclusive have been determined by Hoerr & Ralston (65) in cyclohexane, tetrachloromethane, trichloromethane, ethyl acetate, butyl acetate, methanol, isopropanol, *n*-butanol, nitroethane, and acetone. Some interesting interpretations can be made from their data. The fatty acids generally are more soluble in the lower alcohols than in other solvents investigated except trichloromethane. Except for the alcohols, the fatty acids show a marked correlation between their solubilities and the polarity of the solvent. In the nonpolar solvents, the solubilities of the acids are almost linearly dependent on temperature but deviate as the polarity of the solvent increases. Important relative solubility data on saturated fatty acids and eicosenoic acid, erucic acid, oleic, linoleic, and linolenic acids, at temperatures down to  $-70^\circ$  C. in acetone, methyl alcohol, and Skellysolve B, were reported by Foreman & Brown (66). Although the solubility data are

not absolute because of the difficulty of attaining equilibrium at low temperatures, these data should be extremely useful, as the investigators point out, for separation work involving fatty acids.

Mattil & Longenecker (67) have shown that a linear relationship exists between the composition and the refractive indices of mixtures of highly purified fatty acid methyl esters. The relationship, with its limitations, provides an additional analytical tool for calculation of the composition of unknown methyl ester mixtures. This method augments those methods of calculation based on iodine values and saponification equivalents.

Dutton (68) has applied the use of a differential refractometer in the analysis of colorless compounds separated by chromatographic adsorption. When the method was applied to the resolution of a mixture of stearic and oleic acids, the total recoveries were 99 per cent and 84.8 per cent stearic acid. Application of the method is limited by the requirement that the solvents must have refractive indices differing from those of the dissolved solutes.

#### GLYCERIDES

*Naturally occurring glycerides.*—Henderson & Jack (69) report the fractionation of cow milk fat from solvent at low temperatures into five less complex glyceride mixtures. They have modified the earlier procedures to provide a remarkable degree of reproducibility when different lots of the same fat are employed. Detailed studies of the glyceride composition of the fractions isolated are in progress.

The low temperature crystallization of glycerides has been extended in Hilditch's laboratory to a study of herring oil by Bjarnason & Meara (70). In contrast to whale oil, herring oil contains unsaturated acids of the  $C_{16}$ ,  $C_{18}$ ,  $C_{20}$ , and  $C_{22}$  series with no one acid group predominating. This fact considerably complicates the problem of glyceride structure analysis and makes it difficult to determine with certainty how each group of homologous acids is distributed among the glycerides. It is possible to conclude, as the authors have done, that about one third of the oil consists of glycerides containing only unsaturated acids, and that about 60 per cent of the glycerides had one saturated and two unsaturated acyl groups: of the latter about half had only mono-ethenoid acids and the other half had both mono- and poly-ethenoid acids.

Hilditch & Meara (71) reported on a solid seed fat, niam fat, from the kernels of *Lophira alata* (family Ochnaceae). The mixed fatty

acids included about equal proportions of the saturated acids (myristic, 1.9; palmitic, 27.1; behenic, 14.2; and lignoceric, 2.3 per cent) and unsaturated acids (tetra- and hexadecenoic, 1.5; oleic, 14.5; linoleic, 33.3; and docosenoic, 5.0 per cent). Interestingly enough, no stearic or arachidic acids could be detected. Glyceride structure analysis showed that more than 80 per cent of the fat had at least one linoleic acid group per triglyceride molecule; half of the molecules included one oleyl group and somewhat less than half were monolinoleyl disaturated triglycerides; and 9 per cent of the triglycerides were mono-saturated-dilinenins.

Jackson & Longenecker (75) analyzed a sample of babassu oil for both fatty acid and glyceride composition. Of special interest was the finding that the component fatty acids were distributed approximately at random throughout the glycerides. If all the saturated acids in the oil had been randomly distributed there would have been 65.2 per cent, on a molar basis, of trisaturated glycerides. The actual amount found was 67.3 per cent. Likewise the determined fatty acid analysis of these trisaturated glycerides was a first approximation of the analysis calculated on the basis of complete random distribution of the component acids of the whole fat.

*Synthetic glycerides.*—Fundamental studies on the properties of glycerides have been made possible by the preparation of several series of synthetic glycerides containing unsaturated fatty acids. With the development of a method for preparing unsaturated acid chlorides of highly purified oleic, elaidic, linoleic, and linolenic acids (10), Daubert and co-workers (73 to 78) have synthesized, by previously accepted methods, the following series of glycerides, in which the saturated acid component was varied in carbon chain length from  $C_8$  to  $C_{18}$ : (a) 1-monosaturated-3-oleyl diglycerides, (b) symmetrical mono-oleyl-disaturated triglycerides, (c) unsymmetrical monoelaidyl-disaturated triglycerides, (d) unsymmetrical monosaturated-di-elaidyl triglycerides, (e) 1-monolinolein and 1-monolinolenin and trilinolenin, (f) unsymmetrical linoleyl-disaturated triglycerides, (g) unsymmetrical mono-saturated-dilinoyleyl triglycerides.

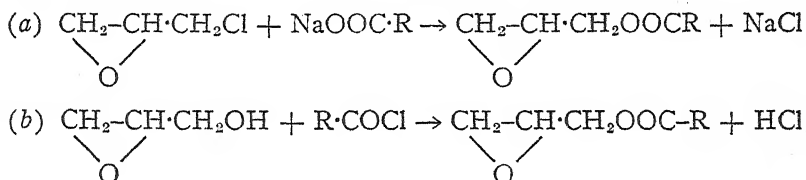
In all instances, the glycerides were of a high degree of purity and their structures were demonstrated by hydrogenation. The melting points and refractive indices both increased numerically with increasing length of the carbon chain of the saturated fatty acids. Spectrophotometric examination of those glycerides containing linoleic and linolenic acids indicated insignificant amounts of preformed conjugated

diene and triene material. It was pointed out by these investigators that while the linoleic and linolenic acids used in their syntheses were obtained by debromination procedures the glycerides prepared from them will be invaluable as reference materials for the study of glyceride structures and properties.

Triacid triglycerides containing a low melting point component acid were also prepared by Verkade (79) using trityl derivatives. By preparing first both unsymmetrical and symmetrical di-acid diglycerides of saturated high melting fatty acids, the low melting fatty acid (butyric or oleic acid) could later be introduced and the resulting tri-acid triglycerides were obtained in a high degree of purity.

Other glycerides synthesized include tri-dihydroxystearin, tri-trihydroxystearin, and mono-dihydroxystearin (57). The products prepared by direct esterification of glycerol and the appropriate di- or tri-hydroxy stearic acids were impure, but they were apparently satisfactory for nutritional studies.

An additional method for the preparation of fatty acid monoglycerides can be recommended on the basis of the synthesis of glycidyl esters of aliphatic acids by Kester, Gaiser & Lazar (80). Glycidyl palmitate was prepared by each of the following reactions:



The glycidyl palmitate is readily hydrated to 1-monopalmitin by cold dilute mineral acid.

In a study of the metabolism of tributyrin and partially hydrogenated linseed oil, Morehouse (81) synthesized the isotopic tributyrin by the saturation of ethyl crotonate with deuterium, transformation to deuterobutenyl chloride, and condensation with glycerol. The deuterium content, while only 75 per cent of the theoretical, was sufficient to follow the fate of the tributyrin in metabolism.

*Special physical studies.*—Daubert & Clarke (82) obtained evidence by thermometric measurements that synthetic symmetrical monoöleil-disaturated triglycerides exhibit polymorphism similar to the fully saturated triglycerides. They have chosen to name these different transition forms by the use of Roman numerals, e.g., Form I,

Form II, etc. Form I always refers to the highest melting form which is usually the form obtained on crystallization of a specimen from solvents. Although this is a departure from the present system of nomenclature, it seems justifiable at least until agreement can be reached among investigators in the field on the best method of naming the different crystalline forms of the glycerides. The temperatures of transition of two polymorphic forms for 1-monolinolein, 1-monolinolenin, and trilinolenin were reported by Daubert & Baldwin (77). It is of interest to note that the transition points of both crystalline forms for 1-monolinolenin are higher than for 1-monolinolein.

Polymorphism studies by dilatometric technique are described by Bailey & Kraemer (83, 84). Low temperature calorimetry has been applied by Bailey *et al.* (85, 86, 87) to a study of the thermal properties of fats and oils. A quick micropenetration technique, applicable as a control in the hydrogenation of fat products, is described by Feuge & Bailey (88) and viscosities and densities of hydrogenated cottonseed oils are reported by Wakeham & Magne (89).

Long spacing and short spacing x-ray diffraction data reported by Filer *et al.* (90) for the isomeric monoglycerides indicate that the 1-monoglycerides have a greater angle of tilt in the hydrocarbon chain than the isomeric 2-monoglycerides. A direct relationship between the long spacings and the melting points of the monoglycerides is indicated. A point of importance in this publication is the completeness of the side spacing data for identification purposes. The same investigators (91) have corrected Malkin's (92) erroneous association of the intermediate melting crystalline form of tristearin with the x-ray diffraction pattern for the lowest melting ( $\alpha$ ) form. They also pointed out that crystalline glycerides obtained by crystallization from solvent do not always exhibit an x-ray diffraction pattern characteristic of the most stable form.

#### PHOSPHOLIPIDS

The method used in the determination of choline is a limiting factor in many phospholipid studies. Each method thus far proposed has been subject to limitations and the modifications of the reineckate precipitation suggested by Enteman *et al.* (93) are not likely to overcome all the earlier objections to the use of reineckate. The new method takes advantage of the increased solubility of ammonium reineckate in 1.2 *N* hydrochloric acid to provide a more concentrated solution of reineckate; it therefore offers a wider range of application.

Complete precipitation of choline is obtained within thirty minutes.

From the same laboratory, a new approach to the separation of phospholipids has been presented. The initial report (94) reveals a convenient method for the resolution of a mixture of liver phospholipids into two groups, one containing choline and the other choline-free. The entire phospholipid mixture is adsorbed quantitatively from a petroleum ether solution on magnesium oxide followed by an elution with methanol of the phospholipids containing choline; or, alternatively, the non-choline-containing phospholipids may be adsorbed by magnesium oxide directly from a methanol solution of the mixture. This method seems certain to find wide application.

In an extensive report on thromboplastin, Chargaff *et al.* (95) have discussed the nature of the lipids contained in this complex lipoprotein. Chargaff (96) was especially interested to determine whether any of the already characterized components of tissue cephalin are responsible for the thromboplastic effect, *i.e.*, the conversion of prothrombin to thrombin. A purified cephalin preparation from beef brain showed a reasonably high degree of activity which Chargaff cautions may have been due to impurities. Phosphatidyl serine, on the other hand, was completely devoid of thromboplastic activity.

In the thromboplastic protein, the lipids are bound so strongly that the exhaustive extraction with alcohol and ether, usually required for their removal, renders the protein insoluble and inactive. Chargaff & Bendich (97) have been able to effect a partial extraction of the lipid without damaging the activity of the protein by using McFarlane's technique (98) of transferring tightly bound cellular lipids to ether in a freezing mixture at  $-25$  to  $-30^{\circ}$ . One third of the total lipid was removed. It represented 18 per cent of the starting material and was rich in acetal phospholipids. The thromboplastic activity of the extracted protein moiety was higher than that of the original material isolated by ultracentrifugal fractionation.

A phospholipid of special interest in connection with serodiagnostic tests for syphilis has received additional attention (99). Pangborn has modified the preparation of the material she has termed "cardiolipin." She now recommends that the active material be extracted from beef heart with methyl alcohol and precipitated from the crude extract with barium chloride. It is finally separated from the cephalin fraction by fractional crystallization of the free phospholipids in acetone.

Kaucher *et al.* (100) have applied methods for lipid distribution



analysis (101) to a series of beef organs and voluntary muscles of various animal species. Total phospholipid was calculated from the values for lipid phosphorus by multiplying by 23.54; choline phospholipid was represented by the ratio between the molecular equivalents of choline to the molecular equivalents of phosphorus, multiplied by the total phospholipid; cephalin was represented by total phospholipid minus choline-containing phospholipid minus sphingomyelin; cerebrosides were calculated from the values for galactose multiplied by 4.55; and neutral fat was calculated by multiplying the value for acetone-soluble glycerol by 9.62. It was observed that the total lipid minus neutral fat ("essential" lipid) was about 50 per cent of the dry weight of brain tissue but only 4 to 8 per cent of the dry weight of stomach and intestines. This "essential" lipid was predominantly phospholipid in all cases although cerebroside was present in all tissues examined except liver. An unexpectedly high concentration of cerebroside was found in cardiac and skeletal muscles. From the distribution of sphingomyelin, which was in high concentration in brain, lung, and kidney, the authors infer its importance in the soft organs of the body to be relatively greater than in skeletal muscles. The very high proportion of "essential" lipid found in the dry weight of the tissues examined lends convincing support to the statement that the lipids appear to be of equal importance to proteins as structural components of protoplasm.

A study of considerable interest has been initiated by Schuwirth (102) who has applied the methods developed by Klenk and his associates to a detailed consideration of the lipids of the human spinal cord. The acetone-soluble fraction, glycerides and cholesterol derivatives, comprised 13 to 20 per cent of the dried substance, ether-soluble glycerophospholipids 19 to 30 per cent, cerebrosides 5 to 6 per cent, and sphingomyelin 1 to 2.5 per cent.

The functional unit of both lecithin and sphingomyelin, phosphorylcholine, has received consideration as a possible intermediate in phospholipid metabolism. Riley (103) has found that phosphorylcholine containing radiophosphorus of high activity (104) disappears rapidly following intraperitoneal injection. The "marked" phosphorus soon appears in the inorganic fraction of circulating blood. On the whole, the phosphorus of phosphorylcholine distributes throughout the tissues practically like inorganic phosphorus injected as phosphate. It is improbable, on the basis of Riley's evidence, that phosphorylcholine is used as a unit in phospholipid synthesis.

In continuation of studies on the lipids of tubercle bacilli, Anderson *et al.* (105) have shown again how important it is to consider the composition of the medium in which bacteria are grown. In the case of tubercle bacilli, glycerol is apparently necessary for the production of certain lipid factors. On a dextrose medium, no phospholipid was present in the human strain, H-37, only acetone-soluble lipids and a low melting wax. The latter contained in addition to mycolic acid some dextrorotatory acids analogous to phthioic acid.

Cell residues remaining from material originally obtained in 1939 in a study of the human tubercle bacillus, H-37, yielded fractions which were in many respects analogous to those isolated earlier from the bacillus itself (106, 107).

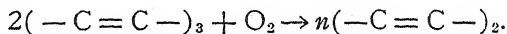
In a brief report on the phospholipids of several grasses, Shorland (27) obtained an appreciably higher yield from freshly-cut cocksfoot, or rye grass, or white clover than from samples stored in alcohol or air dried. Relatively pure lecithin-cephalin fractions were obtained in amounts up to 15 per cent of the total phospholipid but pure samples of lead phosphatide could not be prepared. The component acids were highly unsaturated. Rewald (108) claimed to have extracted the phosphatides and waxes from dried grass using acetone, petroleum ether, and finally alcohol-benzene. Rewald (109) also studied the phospholipid content of petals and stamens from a group of plants.

Two reports have appeared on the chemistry of phospholipids of the soybean (110, 111) and one on the cottonseed (112). Thornton *et al.* (111) obtained a preparation from soybean which was 97 per cent lecithin and 3 per cent cephalin and studied its fatty acid content. It contained a high proportion, 62.9 per cent, of linoleic acid. Olcott's work (112) indicated that the cottonseed phospholipid resembles soybean lipositol more closely than other phospholipids described as plant constituents. Hutt & Weatherall (113) report a system of analytical methods for commercial lecithin.

Bohonos & Peterson (114) report the isolation in crystalline form of an unusual lipid from the mycelium of *Aspergillus sydowi*. The analytical data on the material itself and on its tetraacetate indicate the formula  $C_{46}H_{93}O_5N$ . Degradation of the compound led to the conclusion that the material was identical with fungus cerebrin, isolated by Reindel *et al.* (115), for which the structure  $C_{15}H_{31} \cdot CHOH \cdot CH \cdot (CHOH \cdot CH_2 \cdot CH_2OH) \cdot NH \cdot CO \cdot CHOH \cdot C_{24}H_{49}$  had been proposed. The material could be extracted from mold tissues only after autolysis.

## OXIDATION

Further progress during the past year has been made in furthering our understanding of the mechanism and kinetics of autoxidation reactions. Brauer & Steadman (116) followed the autoxidation of  $\beta$ -eleostearic acid by oxygen uptake experiments in liquid solution with simultaneous determination of the absorption spectra of the autoxidizing solutions. These investigators found that a little less than one-half mole of oxygen suffices initially to destroy one mole of triene system. On this basis their results could be explained by one of two hypotheses; (a) the peroxidation is succeeded by a very rapid decomposition reaction involving the peroxidized molecule and one intact molecule of  $\beta$ -eleostearic acid, or (b) the initial peroxidation may lead either directly or in a rapid secondary reaction to the formation of dimeric products. The absence of low molecular weight decomposition products and the appearance of dimers of  $\beta$ -eleostearic acid as the major product of reaction supports the second hypothesis which may be represented as follows:



After its formation, the dimer then reacts with  $m$  moles of oxygen to produce spectrophotographically inert products capable of absorbing  $(3 - nm)$  moles of oxygen. Complete interpretation of the path of reaction is lacking in the absence of knowledge of the intermediate and final products of the reactions.

Trieb (117), on studying the effects of dilution on the autoxidation of  $\beta$ -eleostearic acid, suggests the following mechanism of autoxidation: (a) dimerization of chains in undiluted systems, in partial agreement with Brauer & Steadman's hypothesis; (b) increasing dilution prevents dimerization and favors the 1,2- or 1,4- addition of oxygen; (c) the 1,4, addition product is rearranged to the 1,4-enol and hydrogenated to the 1,4-glycol, while the 1,2- addition product is split under formation of aldehydes.

In support of Farmer's hypothesis (118) that a hydroperoxide is first formed as the primary product in the oxidation of olefinic bonds, Sutton (119) found that methyl elaidate readily absorbs oxygen at 35° when irradiated by ultraviolet light to form a hydroperoxide and follows the same course of reaction as methyl oleate. Hydrogenation and saponification of the reaction products resulted in a mixture of hydroxystearic acids, one of which was isolated. Sutton, however,

could not determine the position of the hydroxyl group in the isolated acid. Atherton & Hilditch (120) studied the union of gaseous oxygen with methyl oleate at 20° and 120° and confirm the view of Farmer that the autoxidation of methyl oleate at 20° involves the conversion of the methylene group at carbon atom eight or eleven of the acyl chain into a hydroperoxide. Evidence that the temperature of reaction affects its course was obtained when the reaction was carried out at 120°. At this temperature the oxidation appeared to proceed exclusively at the double bond followed by secondary reactions along other points in the aliphatic chain. There was little or no evidence for the formation of hydroperoxide.

The oxidation of drying oils, including theories of the mechanisms and present evidence in support of these mechanisms, has been excellently reviewed in the past year by Hollis (121). Farmer (122) pointed out the dissimilarity of oxidation of rubber and drying oils in that drying oils contain only one methylene group between double bonds. On activation of the methylene hydrogen atoms a resonating system is formed with the production of conjugation. Conjugation in substantial degree has been detected during the oxidation of ethyl linolenate.

Fatty acid esters were prepared from linseed oil, methyl palmitate, and methyl oleate by alcoholysis with furfuryl alcohol and by partial saponification in furfuryl alcohol (123). Although the furfuryl esters contained conjugated double bonds they did not possess any increased drying power, and were distinctly inferior to the linseed oil from which they were made. Heat polymerization of the esters indicated that appreciable reaction occurred at the double bonds of the furfuryl molecule, and that diacylation was also involved, two ester molecules being assumed to split off one fatty acid molecule.

Schwarzman (124) in a study of drying oil films obtained experimental evidence that the primary reaction in their oxidation is the formation of peroxides which arrange themselves into hydroxylated ketones. After the induction period the peroxides catalytically accelerate further oxidation. The evidence is based upon the presence of peroxides and hydroxyl groups, and yellowing of the oxidized films which indicated the presence of polyketones. In support of the general theory of polymerization of drying oils their evidence indicates that conjugation precedes polymerization.

Polyhydric alcohol esters of the unsaturated fatty acids in soybean and linseed oil produced faster drying, quicker bodying, greater water

resistant, greater age resistant, harder and tougher film products than the glycerol esters (125).

Two publications have demonstrated that for a given oil a close relationship exists between the iodine and thiocyanogen values and the proportions of oleic, linoleic, and linolenic acids. Sallous & Sinclair (126) working with linseed oil and Scholfield & Bull (127), working with ninety-five samples of soybean oil which varied in iodine value from 99.6 to 147.6, each observed a straight line relationship between the thiocyanogen value and the iodine value and were able to express the percentages of the individual acids as a function of the iodine value alone. The prediction equations listed below may be used for estimating the component acids of linseed and soybean oils:

Acid Per cent	Linseed Oil (126)	Soybean Oil (127)
Saturated =		$20.5 - 0.045 \text{ I.V. (s.e} = 0.9)$
Oleic =	$-0.48 \text{ I.V.*} + 113.08 \text{ (s.e} = 1.06)$	$128.3 - 0.792 \text{ I.V. (s.e} = 2.5)$
Linoleic =	$0.055 \text{ I.V.} + 0.73 \text{ (s.e} = 1.80)$	$-31.9 + 0.669 \text{ I.V. (s.e} = 3.2)$
Linolenic =	$0.49 \text{ I.V.} - 37.93 \text{ (s.e} = 0.88)$	$-17.0 + 0.170 \text{ I.V. (s.e} = 1.5)$

\* I.V. = iodine value.

Several investigators have reported fatty acid composition data on seed oils which offer possibilities as substitutes for linseed oil. Hilditch (128) reported fatty acid analyses on a group of vegetable seed fats not previously reported. Among these was alfalfa seed oil which had a very high content of linoleic and linolenic acids. Hemry & Grindley (129) have also called attention to a group of oil bearing seeds grown in the Sudan which have relatively high proportions of linoleic acid. The drying properties of these oils were confirmed by oxygen absorption tests.

The effect of metallic salts on the drying of linseed oil has been studied by Rudd (130). Metallic salts influence the drying of linseed oil, the effect depending on the drier used and the nature of the salt. The general effect produced is a prolonging of the induction period and a decrease in the rate of oxygen absorption. The effect of the metallic salt is not due to a permanent irreversible poisoning of the metal drier. Sodium sulfate and sodium phosphate have no effect on linseed oil in the absence of drier. Rudd interprets the inhibition of drying in terms of the surface action of the salts.

For the purpose of enhancing their value as drying oils it has long been desirable to find methods of inducing conjugation in fatty oils containing unsaturated acids. The use of siliceous materials for this

purpose has been previously described by Turk & Feldman (131). Recently the use of metallic oxides has been investigated by Turk & Boone (132). They found that conjugation could be induced in linseed oil fatty acids by the catalytic effect of metallic oxides. The catalytic effect was greater for the oxides of higher molecular weight. While the same catalysts and techniques could be applied to the oil itself, the direct isomerization of the oil is more difficult and the magnitude of the effect is less.

#### ANTIOXIDANTS

In parallel with recent research on the mechanisms of the autoxidation of fats, investigations on antioxygenesis, particularly as related to edible oils and fats, have been focussed on the development of materials which will function adequately as inhibitors of autoxidation. A great deal of activity has been in evidence during the past year. Much of it has been related to the efficiency of various organic compounds as stabilizers, although it is possible that an over-emphasis has been placed on the value of those antioxidants which are effective in accelerated tests. There is without doubt a dearth of information as to the mode of reaction of inhibitors of oxidation. This situation has led to an indiscriminate use of the term antioxidant to include not only the materials which are directly involved in the oxidation reaction but also those which function synergistically in the stabilization of fats. Complete clarification of the terms antioxidant and synergist are not possible; for this reason all those substances which prolong the induction period in accelerated tests are considered together here.

Many phenolic substances have the ability to inhibit the autoxidation of fats at relatively low concentrations. In general, however, the most effective are those which have some type of oxygen linkage in the ortho and/or the para position to the hydroxyl group. The antioxidant activities of these phenolic substances, however, are enhanced by a wide variety of compounds, such compounds functioning as synergists. Nordihydroguaiaretic acid, synthesized in 1918 from hydroguaiaretic acid ether, and isolated recently from a common desert bush, has been shown by Lundberg *et al.* (133) to have excellent antioxidant activity, particularly in the stabilization of lards. Its activity is enhanced in the presence of ascorbic acid. It is more soluble in fats than hydroquinone but less soluble than the tocopherols.

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steam lard. In general, however, the same materials were more effective with vegetable oils than with lard, possibly because of the presence of natural inhibitors in the vegetable oils with which they functioned synergistically.

Other compounds whose function as antioxidants has been studied include pyrogallol, quinone, 1:5-dihydroxynaphthalene, gallic acid and its esters, pyrocatechol and hemotoxylin. Lea (135) found approximately the same relative potencies for these inhibitors but the degree of protection afforded by a single concentration of the inhibitor appeared to be greater at 37° than at 100°. The lower esters of gallic acid proved most satisfactory in affording protection without discoloration. This is in agreement with the results of Boehm & Williams (136) and Mattil, Filer & Longenecker (137) in their work with propyl gallate. The latter investigators studied the action of a group of compounds on vegetable fats and found that the most effective single compound was gallic acid, although *nordihydroguaiaretic* acid, ascorbic acid, and ascorbyl palmitate each about doubled the keeping time of a vegetable oil or fat. They also found that phosphoric acid and citric acids both functioned as synergists in combination with *nordihydroguaiaretic* acid. Filer, Mattil & Longenecker (138) found that the rate of disappearance of gallic acid was independent of its initial concentration in the oil substrate and that gallic acid completely disappeared at the end of the induction period. The rate of disappearance of ascorbic acid during the induction period was also measured. Unlike gallic acid, ascorbic acid was not completely oxidized at the end of the induction period. Further studies on the kinetics of the antioxygenic synergism of quinones with ascorbic acid in fat systems have been described by Calkins & Mattil (139). Other substances found to prolong the induction period of fats include maleic and fumaric acids and their esters (140, 141).

An appreciable increase in the stability of lard as measured by the active oxygen method may be effected by the addition of relatively small proportions (1 to 10 per cent) of various vegetable oils. Riemenschneider *et al.* (142) conclude that the increased stability seems to be related to the tocopherol content of the added oils. Since the tocopherols function most efficiently at lower levels of concentration, the tocopherols added by means of the vegetable oils appear to be capable of reacting synergistically with *d*-isoascorbyl palmitate and commercial lecithin. The conclusion of Riemenschneider *et al.* regarding the efficiency of tocopherol at lower levels of concentration coincides with

that of Bailey and co-workers (143, 144) in their studies on molecularly distilled peanut oil antioxidants and  $\alpha$ -tocopherol. These investigators concluded that both  $\alpha$ -tocopherol and peanut oil antioxidants were effective stabilizers of lard in concentrations of approximately 0.06 per cent or less. With increasing tocopherol concentration, the peroxide level at which rapid oxidation begins was found to increase constantly, whereas the initial rate of peroxide formation passed through a minimum. As Bailey *et al.* point out, this fact accounts for the antioxidant effect of low concentrations of tocopherol. Thompson & Steenbock (145) demonstrated that small additions of  $\alpha$ -tocopherol had no effect on the induction period of either plant or animal fat as represented by cottonseed and soybean oils and lard and oleo oils. If the fats were freed of antioxidant by chromatographic adsorption, the antioxidant effect of  $\alpha$ -tocopherol was demonstrable. On the basis of the observations of Riemenschneider *et al.* and Bailey *et al.* the above results verify the fact that a certain maximum concentration of tocopherol provides the most efficient antioxygenesis. Thompson & Steenbock further found that  $\beta$ -carotene was a pro-oxidant in oils in which the natural antioxidants were first removed by chromatography and that the pro-oxidant effect was greater with plant than animal fats.

The stabilizing effect of *l*-ascorbyl palmitate,  $\alpha$ -tocopherol, hydroquinone, and phospholipids on carotene in cottonseed oil solutions was demonstrated by Williams *et al.* (146). It was found that a tocopherol was only effective in stabilizing carotene in cottonseed oil when the oil was first stabilized with a phospholipid-hydroquinone combination.

Doubtful value is attributed by French & Lundberg (147) to the "chlorophyll test" described by Coe (148, 149) as an indicator of autoxidation in fats. They obtained little evidence of a stoichiometric quenching between chlorophyll and acceptor substances in fats, and also found little correlation between peroxide values of fats, as they are normally obtained, and the amount of chlorophyll fluorescence in the fats they examined.

Spectrophotometric and colorimetric methods for the quantitative determination of gallic acid, added as an antioxidant to fats and oils, have been reported by Mattil & Filer (150).

Successful use of 2,6-dichlorophenolindophenol reagent in acetone for the determination of fatty acid esters of *l*-ascorbic and *d*-isoascorbic acids in fat and oil substrates has been described by Turer & Speck (151). The method can be used for the determination of the ascorbyl palmitate in concentrations of 0.1 and 10 mg. per gram of sample

(0.01 to 1 per cent). The presence of tocopherol or phospholipid does not interfere with the determination. This method and those of Mattil & Filer (150) are valuable in studying the rate of loss of antioxidants added to fats and oils.

Banks (152) described a method of testing the effect of antioxidants on the oxidation of unsaturated fatty acids which more nearly approximate real conditions. It has been indicated previously that the efficiency of antioxidants is affected by the conditions of testing. The method of Banks takes advantage of the catalytic oxidative effect of hematin in linoleic acid, thus making it possible to carry out a rapid oxidation of the linoleic acid at ordinary temperatures and in the presence of water. The use of hemins was reported by Simon *et al.* (153) in a study of the inhibition of catalyzed oxidation of linoleic acid. In a preliminary attempt to correlate antioxidant activity with the structure of the compounds, Banks concluded that substances containing a  $-\text{COOH}$ ,  $-\text{CO}$ , or  $\text{RCO}(\text{OH})$  group are not effective antioxidants.

#### GENERAL

Selachyl alcohol has been synthesized by Baer *et al.* (154) in order to make available a pure product for metabolism studies. This material occurs more widely in nature than other glycerol ethers. It has been isolated previously only as an oil although the constitution and configuration have been proved. The products obtained by Baer *et al.* were *d*- and *l*-selachyl alcohol, m.p. 48.5 to 49.5°, and *dl*-selachyl alcohol, m.p. 46.5 to 47.5°.

Buu-Hoi & Cagniant (155) describe reaction products from a combination of phosphoric anhydride with high molecular weight alcohols of chaulmoogra oil and their hydrogenated products. In an earlier publication, Buu-Hoi *et al.* (156) found that the esters of chaulmoogric and hydnocarpic acids could be reduced to the corresponding alcohols.

Esterification of fatty acids with 1,2-epoxides was found by Fraenkel-Conrat & Olcott (157) to take place readily in aqueous solution when catalyzed with alkali hydroxides, neutral salts, and halides which yield hydroxyl ions with excess reagent. This reaction provides a simple method for preparing monoesters of lower fatty acids with 1,2-glycols. Carson & Maclay (158) prepared a series of fatty acid esters of xylitol, including the pentapropionate, -butyrate, -laurate, -myristate, -palmitate, and -stearate. For determination of purity it was necessary to obtain the saponification equivalents of the esters by

refluxing in *n*-butyl alcohol and potassium hydroxide for two hours.

An interesting observation that solutions of  $\beta,\beta'$ -dichloroethyl sulfide gradually decreased in toxicity led Robertson *et al.* (159) to study the oxidation of thio-ethers by the peroxides of unsaturated fatty acids. This study appears to be particularly pertinent in view of the biological implications of such reactions.

Hydrolysis of sulfated oleic acid produced not only 9- and 10-hydroxy stearic acids but also other isomeric hydroxy acids (160). The preparation of the dimethyl ester of 1,14-tetradecane dicarboxylic acid from the non-volatile, water-insoluble portion of the oxidation products of methyl monohydroxy stearate, indicated that esters of dibasic acids having more than ten carbon atoms were present.

Improvements in the method of preparing perbenzoic acid enabled Swirn *et al.* (161) to prepare for the first time  $\Delta^{9,10}$ -epoxyoctadecanol, m.p. 54 to 54.5°, and a mixture of 9,10- and 10,9-chlorhydroxy-octadecanols, m.p. 61–62°, by epoxidation of oleyl alcohol and subsequent treatment with dry hydrogen chloride in anhydrous ether. A series of  $\beta$ -hydroxy- and  $\alpha$ -keto aliphatic acids were prepared by Adickes & Anderson (162); the lauric acid derivatives melted at 70 to 70.5° and 56 to 56.5°, respectively.

#### REVIEWS

The bibliographical reviews of literature on fats, oils, and soaps by Piskur (1, 2) are again of broad scope including sections on technical treatment of fats, fat products, deterioration, biochemical aspects, characteristics, and composition of lipids and detergents. A new abstracting service (163) which emphasizes the chemical, physical, and technological aspects of fats, oils, and soaps was initiated this year. Smedley-Maclean (164) has contributed a brief monograph on fat metabolism. Other interesting and valuable reviews are those by McHenry *et al.* (165, 166), Goldsmith (167), Daubert (168), and Kaufmann (169).

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# THE CHEMISTRY OF THE AMINO ACIDS AND PROTEINS

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The prolongation of the war has affected the volume and scope of published work in this field by occupying the time of many investigators with problems of practical interest to the military services, and by delaying the publication of many results. Progress in the chemistry of the amino acids and proteins is largely in directions well marked out before the war. The problem of protein denaturation continues to be studied by those whose interest centers on the configuration of polypeptide chains and the existence of labile linkages in the native protein molecule. The possibility of drawing inferences about structure animates much work on the modification of protein specificity, or activity, by mild and more or less specific chemical treatments, such as alkylation. Interest in the striking interactions of proteins with other large molecules, including synthetic detergents, continues to result in the discovery of new phenomena which promise to illuminate both the theory of protein structure and the laws of macromolecular physical chemistry. Work on the fibrous proteins is proceeding at an increasing level of activity. The usual large volume of work on new protein preparations and on their composition and properties has been reported. As a result of the war and of mass production methods, many of these studies are concerned with the human plasma proteins. Work on molecular weights and shapes of protein molecules, and on their thermodynamic functions, shows some diminution, but interesting new methods of studying solvation have been reported. No effort has been made in this review to deal with papers which are predominantly of medical, immunological, or industrial interest, and no mention is made of those aspects of the chemistry of amino acids and proteins (proteolytic enzymes, protein and amino acid metabolism, x-ray studies) which are treated fully elsewhere in this volume.

The year marked the initiation of an excellent new annual publication (1) which is to be devoted to providing comprehensive and critical reviews of special topics in this field. Chapters on protein hormones (Chow), the structural proteins of cells and tissues (Schmitt) and of muscle (Bailey), lipoproteins (Chargaff), nucleoproteins (Green-

stein), immunology (Treffers), and the combination of proteins with alkaline earths (Greenberg) are included. The current volume of another publication (2) contains articles on transamination (Herbst), on the antibacterial peptides (Hotchkiss), and on certain enzymes (Pigman, Nelson, and others). Two articles which review our knowledge of protein synthesis and which speculate on reaction mechanisms have recently appeared (3, 4). Astbury, in a volume on the biological aspects of colloid chemistry, has reviewed the x-ray evidence concerning protein structure, and the relation of x-ray evidence to chemical evidence (5). A critical review of the literature dealing with protein denaturation has discussed at great length the bearing of the detailed knowledge of this very complicated and still obscure phenomenon on the question of the structure of the intact molecule (6).

Biochemistry in general, and the field of amino acid and protein chemistry in particular, has suffered a great loss in the death of Max Bergmann at the height of a notably productive career. Current ideas of peptide synthesis, amino acid analysis, enzyme-substrate relationships, the specificity of the proteolytic enzymes, and theories of protein structure, have been more profoundly influenced by the work of Max Bergmann than by that of any other organic chemist since Emil Fischer.

#### PROTEIN PREPARATIONS AND THEIR COMPOSITION

*Preparation and crystallization of proteins.*—Methods have been described for preparing large crystals (up to 150  $\mu$  long) of seed globulins, egg albumin, and muscle albumin (7). The crystallization of some of the proteins of human muscle has been reported (8). Crystalline albumin has been isolated from chicken blood (9). The gonadotropin from serum, a protein of high carbohydrate content, has been purified further and partially characterized (10). The cholinesterase of serum has been concentrated (11), and a crystallized mucoprotein with high cholinesterase activity, and which contains neither sulfur nor phosphorus, has been isolated (12). A new flavoprotein enzyme which oxidizes *l*-amino acids has been isolated from rat kidneys (13). A globulin of molecular weight 430,000 has been isolated from the horse chestnut, but has not been entirely freed of a higher molecular weight impurity (14). The preparation of crystalline horse-radish peroxidase (15, 16) and of horse liver catalase (17) has been described. An iron-protein complex, differing from ferritin, has been

isolated from liver (18). Diplococcin, a new thermostable, water-soluble antibacterial protein or polypeptide, has been separated from streptococci grown in milk (19).

Reference is made throughout this review to results of work on the plasma proteins, carried out as an integrated program for the military services. Brief reviews of parts of this work have appeared (20, 21, 22), and a group of twenty-three related chemical, physico-chemical, and immunological investigations have been published in a single issue of the *Journal of Clinical Investigation*. Several references (23 to 27, 71, 199, 200) are to papers of this group. The background studies in large-scale fractionation, characterization, and stability have made possible a steady supply, to the armed services, of the following standard, stable preparations from human blood: whole plasma, fibrinogen, thrombin, fibrin films, fibrin foams, immune globulins, isohemmagglutinins, and albumin.

New studies on the thrombin activity of the globulins of several species (28), on prothrombin (29), and on Bence-Jones protein (30, 31) have recently appeared. Significant differences between the serum fractions obtained electrophoretically, and the corresponding fractions obtained by sodium sulfate precipitation, have been reported (32). Experiments on the use of despeciated bovine serum (33), and of protein digests (34, 35), as clinical substitutes for plasma or whole blood have been reported.

*Prosthetic groups of proteins.*—With the exception of a paper on carboxylase (36), recently published studies of the relationships between proteins and their prosthetic groups have been concerned with hemin derivatives. The absorption spectra of hemoglobin and its derivatives in the visible and near infrared regions have been investigated in some detail, to form the basis of a means of quantitatively determining carbon monoxide hemoglobin (37). Similar data for myoglobin and a number of its derivatives have been published (38). Equilibrium constants for the reactions of methemoglobin with fluoride, cyanide, and thiocyanate have been determined, and the kinetics of the reaction with cyanide has been studied as a function of pH. The reaction velocity is greatest at the isoelectric point of the protein (39). The claim that myoglobin is reversibly transformed into cytochrome by treatment with hydrosulfite and a pyridine-hydrazine reagent (40) continues to bring forth new critical replies. One of these (which is sufficiently conclusive) is based on analytical data (41); the other questions the homogeneity of the myoglobin used, and offers an alter-

native explanation of the spectroscopic observations on which the claim was based (42).

The application of the common term "hemochromogen" to compounds formed by heme either with organic bases of low molecular weight or with certain native and with all denatured proteins, has long implied an analogy between the two types of compounds. It is now believed that both involve linkage with the iron atom; those formed by native proteins (other than globin) are stated to be analogous to caffeine-heme and not to involve the iron atom; only globin and the protein portions of certain enzymes are capable of combining with both the porphyrin and iron (43). Theorell has deduced the mode of combination of hemin with horse-radish peroxidase protein by means of a quantitative comparison of the titration curves of the combined and uncombined protein (44). He concludes that the iron atom is attached to one carboxyl group of the protein, and that a carboxyl group in hemin is combined with a group in the protein which has a  $pK$  of 10.2. The protein component has normal activity when combined with protohemin, 53 per cent activity when combined with mesohemin, and 62 per cent activity when combined with deuterohemin. Efforts to form active preparations by combining the protein with eight other hemin derivatives did not succeed (45). The magnetic properties of the intact enzyme and of some of its derivatives have been determined under a variety of conditions, and correlated with concurrent changes in the absorption spectrum (46). Similar measurements have also been made with crystalline horse-liver catalase (47).

*Composition of proteins.*—The mathematical consequences of the periodicity theory of Bergmann & Niemann have been examined by Ogston. It is shown that two and three are not unique as prime factors which can produce a regular array of the type postulated by the periodicity theory (48). If these factors alone are shown to occur with natural proteins, they must be regarded as clues to either a unique type of configuration or mode of formation. The significance for theories of protein structure of the products of the partial hydrolysis of proteins has been discussed in a recent review (49).

The determination of the composition of proteins, once preparations of reliable reproducibility and homogeneity become available, encounters problems at two stages. The first is to hydrolyze the protein without producing secondary changes. The second is to quantitatively determine the amino acids (or peptides) in the hydrolysate, either by isolation or by other procedures. Only one new nonenzymic

matic method for hydrolyzing proteins, involving the use of metallic ions as catalysts, has been described (50). Enzymatic hydrolysis is considered in another article in this volume (p. 31). However, a few papers concerned with the extent to which free amino acids are liberated by digestion with various enzymes (51, 52), and with the average size of hydrolysis products when the digestions are carried out in various ways (53, 54), may be referred to here. The results of the last study (on casein) corroborate ultracentrifugal evidence presented some years ago (55, 56) that hydrolysis by proteinases in acid solutions rapidly degrades a relatively small number of molecules at a time to an average size corresponding to tetra- or pentapeptides. It is reported that concentrated solutions of dried bovine serum resist tryptic digestion, but become digestible in the presence of 20 per cent ethanol (57). This observation is consistent with the theory of Linderstrøm-Lang that the concentration of denatured protein, in constant equilibrium with native protein, is a prime rate-determining factor in enzymatic hydrolysis.

In the analysis of hydrolysates, progress has been made in the application of chromatography, using paper as the adsorbent for purely qualitative analysis (58), and using sulfide-activated charcoal for isolation (59). New methods of determining the amino acids include the use of 3,4-dichlorobenzenesulfonic acid for estimating histidine (60), an enzymatic method for glutamine (61), and microbiological methods, directly applicable to hydrolysates, for glutamic acid (62) and leucine (63). Arylsulfonic acids, already familiar as reagents for isolating the basic amino acids, have been applied to isolating dipeptides (64). Data are given for the solubility of several arylsulfonic dipeptides. On hydrolyzing silk fibroin in concentrated hydrochloric acid at 40° for two days about three times as great a quantity of peptides (mostly dipeptides) as of free amino acids is formed. Both glycyl-*l*-alanine and *l*-alanylglycine were isolated in approximately equal yields.

An ingenious method for isolating and estimating the dicarboxylic acids in proteins, described by Cannon, depends on adsorbing them on an ion-exchange synthetic resin before attempting isolation (65). The values obtained on applying this method to egg albumin, lactoglobulin, and edestin are in fair agreement with those of Chibnall (155), which were based on a refinement of the Foreman alcohol-precipitation of the calcium salts. The figures obtained for the total acids agree within 1 or 2 per cent, which is even better than the agreement for the individual acids. A more rapid method, which requires only one gram of pro-



tein and gives accurate results, is based upon separately estimating total carboxyl groups (by electrometric titration) and  $\alpha$ -carboxyl groups (by the ninhydrin reaction) in the resin-adsorbed fraction of the hydrolysate (66). The difference between the total carboxyl groups and the ninhydrin-reactive groups gives the glutamic acid content. The difference between the ninhydrin-reactive groups and the formaldehyde-reactive groups (determined electrometrically) corresponds to the content of aspartic acid.

Determinations of some of the amino acids of several myoglobins (67), tyrocidine (68), lactoglobulin (69), human milk proteins (70), and human plasma proteins (71) have been published. Although the last study shows significant differences between the corresponding human, horse, and bovine proteins, it confirms the existence of a nearly common amino acid pattern in homologous proteins, as a number of earlier investigations have reported. Determinations of tyrosine and tryptophane in peanut proteins (72), and of the sulfur-containing amino acids in certain seeds (73) and in rat and rabbit tissues (74) are also reported. A new book assembles existing data on the amino acid composition of proteins and natural foods (75).

A peptide which contains *p*-aminobenzoic acid has been found in yeast (76). A sensitive microbiological method indicates that  $\beta$ -alanine is absent in silk, hemoglobin, egg albumin, gelatin, casein, and lactoglobulin (77). The dipeptide, carnosine, which contains  $\beta$ -alanine, has not been found in the peptic and tryptic hydrolysates of muscle (78).

#### AMINO ACIDS AND SIMPLE PEPTIDES

The synthesis of a peptide (*dl*-alanyllalanine) from pyruvylalanine by transamination has been accomplished. As a result, a hypothetical mechanism of biological synthesis of peptides from nonamino acid precursors by alternate amination and acylation reactions is proposed (79). It has been shown that glutamic acid and asparagine react in the presence of air at 100° to produce nicotinamide (80). Nicotinamide also appears to be formed when asparagine reacts with other substances as well. A high molecular weight polypeptide composed entirely of glutamic acid residues has been isolated from the products of *Bacillus subtilis* metabolism (80a).

Synthetic preparations of *dl*-leucine have been shown to be contaminated with isoleucine (81). The synthesis of hydroxyleucine, and its suspected presence in casein, has been reported (82). It is reported

that certain amino acids may be recognized from the color of the alcohol extracts of the decomposition products that result from heating them in a dry test tube until a distinguishable color change appears (83). The absorption spectra of the copper complexes of di-, tri-, and tetrapeptides can be distinguished (84). On the basis of the action of crystalline proteolytic enzymes on angiotonin, conjectures as to the order in which its constituent amino acids are arranged have been made (85).

Considerable new data on optical rotation have appeared (86, 87). The apparent acidic dissociation constants of both homocystine and homocysteine have been determined (88). Hydrogen ion is more tightly bound to both the ammonium group and the thiol group in the longer-chain compound. Analysis is complicated by the fact that the dissociation constants for both groups have nearly the same values. A new and thorough study of the oxidation-reduction potential of the cystine-cysteine system (and of related thiol compounds) has been made (89). Advantage was taken of the fact that in the presence of iodide ion, a reversible one-step electrode reaction occurs. The values obtained ( $-0.27$  volt for cysteine) are in fair agreement with those obtained some years ago by equilibrium measurements with calibrated dyes (90), but depart widely from other published values which were based on rather questionable electrode reactions.

#### THE REACTIONS OF PROTEINS

*Denaturation.*—A recent review (6) has covered this subject comprehensively and only the newest work need be mentioned here. In the review cited above evidence is gathered which confirms the now general impression that with many proteins denaturation is neither a unique, nor an all-or-nothing process, and that the only general manifestations of denaturation are an increase in reactivity of certain constituent polar groups, and a loss of ordered structure or a general tendency to aggregate, commonly manifested by decrease in solubility and increase in particle size. The last probably follows as a direct consequence of the free interaction of the "liberated" groups. The agents causing denaturation are agents that normally affect the equilibria of these polar configurations, and which, to judge by the great effect of all amides, unquestionably include not only the acid and basic groups of the side-chains but also the peptide groups. Recent work, referred to later, has suggested that reactions of certain denaturing agents with the polar groups are innocuous in themselves, but often

initiate disruptive mechanical changes purely because of a steric effect. It has already been shown that analysis of the energy changes accompanying denaturation may lead to a partial identification of the groups which initiate the reactions (91).

On the whole, one is not likely to take exception to the contents of the review (6), except in a few specific details. However, it should be pointed out that if, following the authors, the term denaturation is broadened to include any non-proteolytic change in the molecule, then denaturation does not always abolish specific catalytic or biological activity or many other of the properties which characterize the molecule (92, 93). It is probably of prime importance to distinguish between simple, largely reversible changes in state of aggregation (usually dissociation) which can occur without loss of specific activity, and the more deep-seated changes which are accompanied by high degrees of aggregation, low solubility, loss of specific properties, and which are often only slightly reversible. The potential importance of the distinction lies partly in the probability that the first phenomenon represents an initial step, which often precedes the second, but which in some cases cannot be arrested before the second obscures it. The possibility of arresting the process at the first stage may represent a fundamental difference between the structures of two different kinds of proteins. However, the fact that drastically altered proteins, such as surface spread films, can combine with their antisera (94) indicates the need for caution in attributing large-scale structural significance to all biological specificity or activity. The latter may often be abolished or restored by oxidation or reduction of one or two linkages, or even by combination of one or two groups with a metallic ion.

The increase in reactivity of denatured proteins is by no means a clear-cut phenomenon, and must be specified for each group and each reagent. It cannot even be said that no instances are known of decreased activity caused by denaturation, if enzymatic reactions are included. Certain reducing groups in fibrinogen, which react with iodine, but which are not sulfhydryl, are reported to be as reactive in the native protein as after denaturation (95). The extent of the increase of the reactivity of the sulfhydryl groups has again been shown to depend on the nature of all the ions present when denaturing agents are used (96). The presence of certain sugars is reported to inhibit the liberation of sulfhydryl groups in the heat denaturation of egg albumin (97) although the experiments appear to indicate there was no

combination of the sugars with the protein. It is reported that sulfur is split off not as inorganic sulfide but in organic form when egg albumin is denatured by acid or base (98).

Further studies of denaturation by ultraviolet radiation have been made. The effect on trypsin and papain has been investigated rather qualitatively (99) but more quantitative kinetic studies on trypsin (100) have also been reported. As in earlier work the inactivation followed a first-order law, but the quantum yield appeared to depend on the method of assaying the activity. An effort has been made to show that the rate of denaturation of various gonadotropins in 40 per cent urea is a first-order reaction, but, as in many earlier cases, the results are not clear-cut (101). Of the gonadotropins tested, mare serum gonadotropin alone appeared to be relatively stable in urea. The process of inactivation of catalase at high temperatures has been shown to resemble the denaturation of proteins (102). The effects of radiation, urea, and heat on egg albumin have also been further studied (103).

A series of experiments (104) on the effects of extensive deamination on various proteins shows that they are transformed into much more highly asymmetrical ("linear") molecules, having reduced solubilities and high specific viscosities. The alterations produced by the treatment are too drastic, and the products too inhomogeneous, for detailed conclusions of theoretical interest to be drawn. This objection can also be made to experiments on the effects of ozone on egg albumin (105), which were also reported to change the protein into the fibrous form. An increase in base-binding capacity, the disappearance of tyrosine, and increases in optical rotary power accompanied the change.

Heat denaturation of serum albumin at 70° has been found to result in gradual aggregation of the dissolved molecules, but no conclusive physical evidence, as from measurements of molecular-kinetic constants, of an initial unfolding of the molecule, such as occurs in urea, was observed (106). The physical changes which occur in urea solutions appear to be more drastic, and this is confirmed by the greater susceptibility of urea-denatured albumin to tryptic digestion.

Insulin, heated at pH 2, aggregated into thixotropic, statically birefringent gels, which were shown in electron-micrographs to be composed of fibrils 200 Å wide (107). When disoriented by freezing and thawing, the resulting solution showed stream birefringence, which prolonged treatment or addition of alkali destroyed. The alkali-treated

material appeared to be normal native insulin, and a new heat treatment repeated the cycle.

An analogy to the aggregation of heat-denatured proteins exists in the reported readiness with which proteins in mixtures form stable soluble complexes on heating (108). However, there is evidence that at least some of the complexes observed are formed only in the presence of a multivalent anion (on the acid side of the isoelectric point), or a multivalent cation (on the alkaline side). It is possible that some of the complexes formed are analogous to the more familiar complex inorganic ions. Similar complex formation on heating has been reported earlier on the basis of electrophoretic evidence (109, 110). If the complexes are formed solely as the result of the increased reactivity produced by the process of denaturation, some degree of complex formation should result when the protein solutions are heated separately, and mixed at a somewhat lower temperature. This suggestion is strengthened by the fact that there is electrophoretic evidence for compound formation when denaturation is brought about by ultraviolet light (111).

A study of some of the factors affecting the heat coagulation of several preparations of human serum albumin has been made by utilizing a simple apparatus for measuring the time to produce a given optical effect, which is interpreted as representing a constant degree of aggregation (112). The expected exponential relation to temperature is found, but some of the effects described show that the influence of the ions present on the rate of the "unfolding" stage of the denaturation is not distinguished from their effects on the observed subsequent aggregation equilibrium. Thus, as the authors realize, the method is not suitable for analyzing detailed chemical kinetic effects.

Two new reports of the reversal of heat denaturation of enzymes (invertase and peroxidase) have appeared (113, 114). The experiments with peroxidase indicate that the enzyme is regenerated after heat-inactivation as the result of combination between the insoluble denatured protein and a relatively soluble component, probably the prosthetic group. The apparent reversal of the inactivation of phosphatase by acid has been ascribed to combination and dissociation of the enzyme with fortuitously present heavy metal ions (115).

Superficially related to the reversal of inactivation of enzymes are the claims that denatured antibodies have been regenerated in the absence of the antigen (116). These claims are important in that they imply that antibodies differ from normal serum globulins either in

composition, or in the arrangement of the constituent amino acids in the primary polypeptide chains. They thus tend to disprove the views of Pauling (117) that antibodies are formed by refolding the chains of normal globulins, in the presence of antigen. It has been objected (118) that no valid experimental evidence for the claimed regeneration has been offered, since the particular configurations responsible for the specificity may not all have been destroyed by exposure to the denaturing agent (guanidine hydrochloride). Even if only a small fraction of the treated molecules retain these configurations, the extensive polymerization that always follows denaturation may cause precipitation of a large fraction of the material, in the usual precipitation with the antigen. In this connection it must be recalled that the infectivity of many viruses has been clearly shown to be destroyed by urea (119). To the reviewer the view that the antigen merely guides the refolding of preformed polypeptide chains into a specific pattern appears to discount very heavily known facts as to the constant breaking and resynthesis of peptide bonds in living systems.

Although some of the effects of formaldehyde on proteins are discussed elsewhere, mention must be made of the influence of this substance in denaturation, an effect of importance in the tanning of collagen, in the production of artificial fibers from denatured globular proteins, and in the control of infectivity in immunological procedures. Formaldehyde in low concentrations (2 per cent) does not appear to denature most proteins rapidly (120) although an irreversible effect on the infectivity of tobacco mosaic virus has been reported (121). The addition of formaldehyde to horse serum prevents the usual increase in viscosity brought about by alkali denaturation, and also prevents the appearance of cystine linkages, as measured by the polarograph (122). However, if formaldehyde is added after the addition of alkali the usual effects of denaturation on viscosity are enormously enhanced.

*Modified native proteins.*—The inactivation of enzymes has been commonly treated as evidence of denaturation, and it properly falls within the very broad definition of denaturation offered by Neurath *et al.* (6). However, the apparent reversal of denaturation, sometimes indicated by physical criteria and by certain chemical evidence, often fails to restore biological activity, or leaves it grossly impaired. Likewise, it is well known that activity can often be shown to disappear as the result of mild treatments that do not immediately produce the other familiar manifestations of denaturation. Often, indeed, the ac-

tivity appears to depend on the state of particular groups, such as certain free sulfhydryl or phenylhydroxyl groups, and can be abolished without destroying the configuration of the native molecule (e.g., papain, pepsin, insulin). Thus, a recent careful study has established that the activity of urease (123) can be completely but reversibly suspended by blocking two particular sulfhydryl groups with chlormercuribenzoate, although there are other sulfhydryl groups which appear after the enzyme is treated with guanidine hydrochloride. The reported action of numerous war gases on urease also appears to be due, at least in part, to blocking the critical thiol groups (124). The inhibiting action of traces of certain metallic ions sometimes has the same explanation. Such an explanation appears to account for recently reported observations on the effects of carbon monoxide and cyanide on the adenosine-triphosphatase activity of myosin preparations (125). Here copper is the inhibiting ion.

Earlier work on the acetylation of pepsin with ketene established that the activity of the enzyme survived acetylation of the free amino groups of the molecule, but was progressively impaired or abolished as the hydroxyl groups of the more numerous tyrosine residues became acetylated (126). This result has now been confirmed, using the synthetic pepsin substrate, carbobenzoxy-*l*-glutamyl-*l*-tyrosine, to measure the activity (127). The same synthetic substrate, as well as casein and serum albumin, has also been used in testing the effects of malonylation (produced by treatment with carbon suboxide) of the free amino groups and phenylhydroxyl groups of this enzyme (128). The enzyme lost activity progressively as malonylation proceeded until it disappeared when three quarters of the phenylhydroxyl groups had reacted. Mild hydrolysis, which did not remove the malonyl groups from the  $\epsilon$ -amino groups of the lysine residues, restored the activity. The observation was made that the hydrolysis of serum albumin by pepsin proceeds about 20 per cent further if the substrate protein has itself been malonylated.

Carbon suboxide and phenylcyanate, unlike ketene, have been shown to react even more rapidly with the thiol groups of egg albumin (129) than with the amino and phenolic groups, but the thio esters formed are readily hydrolyzed by base. Difficulties arise in interpreting the effects of any of the reagents which have been used, due to their common lack of specificity. Thus propylene oxide, ethylene oxide, and epichlorohydrin react readily with proteins (egg albumin and  $\beta$ -lactoglobulin) in aqueous solution at room temperature, esteri-

ifying the carboxyl groups and shifting the isoelectric point drastically (up to three units toward the alkaline side), but reaction with amino, thiol, and phenolic groups occurs simultaneously (130). Up to 120 moles of reagent combine per mole of protein, and most of the material combined cannot be removed by exposure to acid or base.

The iodination of various proteins has been reported to yield material possessing some of the biological activity of thyroglobulin. It is claimed (131) that the activity of iodinated casein can be completely accounted for by the amounts of thyroxine which can be isolated, or partially isolated, from an alkaline hydrolysate of the treated protein. However, the thyroxine-containing preparations which result from iodination of casein are stated to lack most of the desirable biological properties of a true thyroid protein (132).

A curious modification of a much-studied protein, egg albumin, showing none of the common manifestations of denaturation, is brought about solely by time (133). The  $A_1$  and  $A_2$  components of the crystalline preparations, the existence of which was earlier demonstrated by electrophoretic measurements, are now shown to be present in quantities which depend upon the age of either the dry crystalline preparations or of solutions. In the course of a year  $A_1$  appears to be transformed wholly into  $A_2$ . It is pertinent to inquire what effect the age of such crystalline protein preparations may have on the dependence of their solubilities (or the lack of dependence sometimes claimed) on the amounts of solid phase present.

*Interactions with large ions.*—Early indications of the strong tendency of proteins to form tightly bound combinations with other large ions (cytological staining, the acid dyeing of wool, isolations carried out by precipitating with certain dyes) were obscured by the fact that the compounds formed were insoluble, and the phenomena observed were often explained as the mutual precipitation of oppositely charged colloidal particles. With the recent great interest in the dispersive properties of synthetic detergents, attention was soon called to the very powerful denaturing action on proteins (134) of certain commercial preparations of compounds containing large anions. At about the same time it was discovered, as the result of a series of studies of the acid-base equilibria of both wool keratin and egg albumin in solution (135), that all of the thirty-odd large anions studied combined with the proteins, and that the tendency to combine increased with the molecular weight of the ion involved (136). Combination was apparent even when no precipitate was formed, as in the case of the



soluble protein. It was observed that, owing to the affinity of the anion for protein, some acid was combined even on the alkaline side of the usual isoelectric point. Indeed, in the presence of salts, quite large amounts of the anion would combine. This combination with anions on the alkaline side of the isoelectric point has escaped the notice of numerous workers in studies with detergents, and has been implicitly denied by others. The fact that in buffered solutions precipitation of proteins by the salts of large ions is not usually observed (137, 138) does not affect the fact that some combination occurs. Although the precipitation of proteins by large ions can sometimes apparently be regarded as an interaction of colloids of unlike charge, this is not always the case, e.g., the precipitation at pH 7 of tobacco mosaic virus by heparin (139).

The combination of large anions with proteins has gained further significance by the demonstration that the same large ions strongly catalyze the hydrolysis by dilute acid of both peptide and amide bonds in proteins (140, 141). The higher the affinity of the ion for the protein, the greater its catalytic effect. Kinetic analysis of the data shows that the anions, by combining with the protein, shift the basic dissociations of both the amide and peptide groups to a much less strongly acid range (pH 1 to 2) than that characterizing these groups normally, and that the acceptance of a proton by these groups precedes hydrolysis of the adjacent C — N bonds. The existence of this catalytic effect has not always been taken into account in studies of the denaturation of proteins by detergents, and its subsequent reversal (142).

Some of the substances used in the investigation just described can be properly described as detergents but others can not. Up to the present time many observations of large-ion effects have resulted from experiments made with detergents, possibly because, although in low concentrations they may denature and aggregate proteins, they have a convenient dispersive effect on native and denatured proteins when in excess (143, 141). It is unfortunate that the effects observed are very frequently attributed to the detergent properties of some of the molecules studied, rather than to their large size, and to the demonstrated tendency of their ions to combine with proteins.

Recent work has fully confirmed the existence of definite combinations between proteins and the large anions of strong acids. Evidence from equilibrium studies (135) and from sedimentation measurements (143) is supported chiefly by electrophoretic obser-

vations (144, 145, 146). An interesting analysis of the stoichiometry of combination between serum albumin and dodecylsulfate ion has recently been reported (147). In mixtures of the two an electrophoretic component can be recognized, the composition of which corresponds to the total acid-binding capacity of the protein. In solutions dilute with respect to dodecylsulfate another definite component can be demonstrated in which the ratio of dodecylsulfate to protein is only half as great. This smaller component has the normal viscosity of serum albumin while the fully combined component has an abnormally great viscosity. The authors very plausibly attribute the existence of the definite but only partly combined component to the fact that all of the free cationic groups of the intact protein are not accessible to large ions. The fully combined material can only be formed by partially destroying the original structure of the protein. If this explanation is accepted, a partial explanation of the denaturing action of large ions may be at hand. Somewhat similar results have been reported for egg albumin and dodecylbenzenesulfonate (148).

In connection with the catalytic effects of large ions on protein hydrolysis it may be noted that a catalytic effect of dodecylsulfonate on the hydrolysis of fatty acid esters was reported some years ago (149). In addition, the reviewer has observed that the longer chain alkylsulfates catalyze their own hydrolysis. At a constant pH, solutions of the dodecyl- and tetradecylsulfates hydrolyze much more rapidly than do the hexadecyl- and octadecyl- compounds (150). The reported observation that phenylhydrazine oxidized by hydrogen peroxide has a slight proteolytic effect (151) may be an instance of the large-ion catalysis described in this section.

*Acid-base equilibria.*—Two general discussions of the dissociations of the polar groups of proteins have recently appeared (152, 153). Much of the foregoing section bears on the combination of proteins with acid and base, since even the smaller ions of common acids show demonstrable differences in affinity for at least the fibrous proteins (135). Myosin in 0.5 *M* potassium chloride is reported to have a combining weight for acid of slightly over 600 gm. per equivalent and a combining weight for base of slightly over 500 (154). These weights are about one-fifth too low, in comparison with those to be expected from figures for the amino acid composition, a result which has been observed with a number of other proteins (155). The difference is less in the absence of salts (156). Like most other proteins, myosin

has little buffering capacity in the physiological range. Inflection points in the titration curve appear at pH 4.25 and at 10.35. The effects of formaldehyde on the titration curve are quantitatively consistent with the assumption that formaldehyde interacts only with the  $\epsilon$ -amino groups of the lysine residues, in the range of pH studied. No significant differences were observed between the titration curves of the native protein and the protein denatured by heat or alcohol (156). As in similar previous reports, this result must be accepted with caution, since denaturation by acid and base may affect the curves obtained for the "native" protein.

Measurements of the acid and base removed by native and heat-denatured collagen from solutions of varying concentrations of each (heterogeneous equilibrium) have been made by a direct analytical method (157). A shift of isoelectric point from pH 6.3 to 7.5 was produced by denaturation, but no differences in maximum combining capacities were found. This method has also been used to demonstrate that collagen combines with salts, and with formaldehyde (158). Calcium chloride is bound to a greater extent than potassium chloride at any given ionic strength. The combination with calcium causes a contraction of the strips of goatskin used. Similar measurements have been made on the combination of wool fibers with formaldehyde (159). The equilibrium between calcium and serum globulins has also been investigated (160).

The effect of formaldehyde on the alkaline branch of the titration curve of proteins parallels effects obtained with amino acids, and usually ascribed to combination between formaldehyde and amino groups. Polarographic studies of this compound formation have now been extended to include histidine, arginine, and lysine (161). An analysis of the equilibrium with histidine and arginine has resulted in agreement with the earlier conclusions of Levy. However, contradicting Levy, it appears that although in arginine two molecules of formaldehyde combine with the guanidine group, in the case of lysine one molecule of formaldehyde combines with the  $\epsilon$ -amino group and one with the  $\alpha$ -amino group. The small amount of combination between formaldehyde and proteins which can be observed on the acid side of the isoelectric point is ascribed to a reaction with the amide groups of the glutamine and asparagine residues (162).

Two papers describing experiments with wool keratin and mixtures of pairs of acids make use of the concept of combination with anions to analyze the complex equilibria which determine the ratio

in which the two acids combine with the protein (163). An alternative method of analyzing the combination of fibrous proteins with acids, which takes into account potential differences at local sites which arise as the result of the combination, has been applied by Gilbert & Rideal (164). Studies on the acid-base equilibria of amino acids in media of lowered dielectric constant have been enlarged by measurements made in 20 per cent dioxane-water mixtures (165).

Wool keratin has been shown to combine with very large amounts of weak acids in the undissociated form (166). When buffer solutions having the same pH, but varying in concentration, are used the amount of acid combined is proportional to the concentration of the undissociated form. The thirteen weak acids employed in this investigation varied in their tendency to combine with the protein over a 300-fold range. Reasons are given for believing that this combination is a case of solvation, and that it involves displacement of the normal combined water. This study suggests a new method for studying hydration, and also implies the possibility that in all but the most dilute buffer solutions the molal volume of the solvated protein may be affected by the buffer employed and its concentration.

#### FIBROUS PROTEINS

New analyses for the basic amino acids, the dicarboxylic acids, and the methionine proline of human hair have appeared (167).

Models for the structure of silk fibroin,  $\beta$ -keratin, and collagen have been proposed in a recent review (168) on the basis of the x-ray evidence and analytical data. Most new studies of structure by means of x-rays are concerned with the long-spacings of keratin and collagen, from 95 Å in feather keratin to 680 Å in collagen, and with the effects of combined substances such as water on these spacings (169, 170). An excellent correlation between the x-ray evidence and the direct measurement of these periods in collagen with the electron microscope has been shown to exist. A similar correspondence between the average observed width of clam muscle fibrils and the spacings deduced from x-ray diffraction has been found (171).

New ideas about structure have been proposed as the result of analyzing the effects produced by treatment of collagen and gelatin with methylsulfate (172). The expected esterification of the carboxyl groups occurs, but it is reported that peptide methylation also occurs, and, in addition, sulfate is bound covalently. To account for the latter effect it is proposed that these proteins contain oxazoline rings formed

by the condensation of the hydroxyl groups of serine and threonine with peptide groups. On treatment with methylsulfate these rings are broken, the enolized peptide is methylated, and the sulfate combines with the liberated hydroxyl group. Methyl halides esterify only and do not produce this additional effect. Efforts have been made in other studies of the methylation and acetylation of fibrous proteins to distinguish between the accessibility of different categories of groups to the agents used (173). The mechanism of the action of sulfites on wool has also been investigated by this method. After treatment of wool first with sulfite and then with methylsulfate, methylocysteine has been recovered from the hydrolysate (174).

The conversion of globular proteins to the fibrous form by heating and stretching has been previously reported (175, 176). This work has now been extended to include other proteins, and treatment by a large number of chemical agents (exposure to very diverse denaturing agents, followed by application of shear stress) (177). The best diffraction patterns are produced when treated protein filaments are drawn to from three to five times their initial lengths, but good results cannot be achieved unless the chemical treatment produces a fiber having a high degree of internal friction and cohesion before drawing.

Since the early investigations of Speakman over fifteen years ago, the stress-strain curve of keratin fibers has been extensively used as a means of comparing the effects of various treatments on the linkages responsible for the cohesive and elastic properties of the fiber structure (178, 179). A careful analysis of the various methods of making such measurements has now led to a method of obtaining results which are independent of the rate of application of load (180). Consideration of the maximum elongation (18 per cent) which can be obtained without damaging the fibers or transforming  $\alpha$ -keratin to the  $\beta$ -form, leads to the conclusion that Astbury's recently proposed structure for  $\alpha$ -keratin (181) is too condensed. An alternative structure is proposed, and a molecular interpretation of the stretching process offered. Permanent set in stretching fibers, produced by heating, is attributed to the breaking of cross-links (principally disulfide) and the formation of new ones by reactions between some of the polar side chains (182). Combinations of dyes with some of these side chains in the fibers reduces their ability to acquire set. In terms of this interpretation the reduced and alkylated keratin produced by Harris and his collaborators (183), which possesses stable artificial cross links, would also be expected to resist set.

If increased resistance to extension is an indication of new cross-link formation, then 2 per cent formaldehyde forms no new cross links in keratin (179, 184) even after boiling for one hour. That this is not generally true is shown by the wide use of formaldehyde in tanning and in hardening artificial fibers made from denatured globular proteins. Such fibers are not only insoluble, but resist the action of some of the proteolytic enzymes. They are, however, susceptible to the action of papain (185). A comparison of the properties of collagen and deaminated collagen treated with formaldehyde has led to the conclusion that the formaldehyde combined with lysine residues stabilizes the structure but that the formaldehyde combined with arginine or peptide bonds does not (186). It is stated that only those scleroproteins that contain histidine or tryptophane form stable combinations with formaldehyde which are not broken by sulfuric acid (187).

Fibrin, the fibrous protein of plasma, is apparently formed by the end-to-end union of very large numbers of rod-like molecules of fibrinogen (188). The latter has been estimated, on the basis of its flow-birefringence, to have a length of  $900 \pm 200 \text{ \AA}$  (189). Human fibrinogen has found noteworthy uses (190) as a plastic film in neurosurgery (191), and as a hemostatic agent (192).

New measurements on the anomalous viscosity and flow-birefringence of fibrous tissue proteins have been made (193). Extensive evidence has been brought forward to support the view that the fibrous protein, myosin, is identical with adenosinetriphosphatase. Addition of 0.004 *M* adenosinetriphosphate to myosin solutions reduced the flow-birefringence by 48 per cent and caused the appearance of inorganic phosphate in the solution. Only inosinetriphosphate, of the many other compounds tested, produced similar effects (194). The activity was inhibited by salts of copper and zinc, but not by any other of a large number of substances tried (195). When examined electrophoretically and by means of the ultracentrifuge, myosin preparations appeared to consist mainly of a single component, which, on isolation, contained over 90 per cent of the enzymatic activity. The sedimentation constant cannot be given with certainty since it is a function of concentration, but it is large enough to indicate a molecular weight in the millions (196). An earlier report described four components, differing widely with respect to sedimentation velocity, in unfractionated material from rabbit muscle (197). According to this report the sedimentation constants increased as the result of the procedures used in purification.

## MOLECULAR CONSTANTS

*Molecular-kinetic measurements.*—The theory and methods of high-speed centrifugation have been reviewed (198), and extensive measurements on the molecular constants of the human plasma proteins have been reported (199, 200). Preliminary measurements with crystalline phosphorylase indicate that it has a sedimentation constant ( $S_{20}$ ) of  $13.7 \times 10^{-13}$ ; and a diffusion constant ( $D_{20}$ ) between  $3.2$  and  $3.8 \times 10^{-7}$ ; by assuming a partial specific volume of 0.74, these values lead to a molecular weight between 340,000 and 400,000 (201). A small quantity of an impurity of much lower molecular weight ( $S_{20} = 4 \times 10^{-13}$ ) was observed in the sedimentation diagram. The molecular weight of crystalline horse-radish peroxidase has been determined by sedimentation velocity methods to be 44,100, which is in good agreement with its content of hemin (202). Crystalline ferritin, which consists of apoferritin and a complex of apoferritin with iron hydroxide, is inhomogeneous in the ultracentrifuge, but apoferritin is homogeneous ( $S_{20} = 17.6 \times 10^{-13}$ ) and has a molecular weight of 465,000 (203). Species differences are small. Changes in the molecular size of the hemocyanin of *Paludina vivipara* as a function of pH are reported and analyzed (204). A new osmotic pressure determination of the molecular weight of egg albumin gives a value of  $45,000 \pm 2000$ , which is in good agreement with the osmotic pressure values of Bull and the results calculated from measurements of sedimentation and diffusion (205). Electron microscope measurements show that the hemocyanin of *Loligo* has a diameter of 8 m $\mu$ , while the hemocyanin of *Limulus* has a diameter of 17 m $\mu$  (206).

The sedimentation velocity of influenza virus A (PR8 strain) has been measured in solutions of varying density but low osmotic pressure. Additions of bovine serum albumin were used to control the density in order to avoid changes in virus particle size which might occur in solutions of high osmotic pressure. The measurements give lower values for the density of the virus (1.104) than those previously obtained. By combining the density with the sedimentation constant in water ( $S_{20} = 742 \times 10^{-13}$ ), the particle diameter is calculated to be 115 m $\mu$ . This is in good agreement with the value 101 m $\mu$  given by electron micrographs. The considerable difference between the density found in these solutions and that of the dry virus, 1.215, indicates that in solution the particles contain about 66 per cent by volume of water, "greatly in excess of that considered to be associated with

protein molecules as water of hydration but similar to the quantity found in organisms of complicated biological structure" (207). It should be recalled, however, that some protein crystals have been shown to contain as much as 83 per cent water when equilibrated with saturated vapor (208, 209). A detailed analysis of the absorption of water by globular and fibrous proteins and nylon (209) has demonstrated that only about one quarter of the total extent of surface (deduced from experiments with films) of globular proteins is covered with a monolayer of combined water when the protein is equilibrated up to quite high relative humidities. With fibrous proteins the ratio is about 0.4. However, at the highest relative humidities, higher than those required to form the limited monolayer, the attraction between adjacent protein molecules, pushed apart by water, is so greatly reduced that more water can easily enter. The protein tends to go into solution and changes in the heat of absorption of water vapor appear. The authors argue, however, that this additional water is not to be included in the water bound by a protein molecule in solution. The most recent estimates of the latter have been based on comparisons of intrinsic viscosities and diffusion constants (210). Consistent with earlier values, these indicate that the water combined with dissolved proteins amounts to about 21 per cent of the weight of the protein.

A preliminary report has been made on particle size distribution, as determined in the ultracentrifuge, in solutions of degraded bone gelatin which have been proposed as transfusion fluids. Fraction-molecular weight curves are given for five stages of hydrolytic degradation (211). Somewhat similar data have been obtained by the method of precipitation-titration, i.e., fractionation by the gradual addition of a nonsolvent, such as acetone, to an aqueous solution (212). This very general method, first developed by Brønsted (213), has been widely applied in recent years to the analysis of synthetic polymers and of mixed degradation products of natural products. Efforts have been made to apply the method to degraded casein, edestin, and egg albumin, as well as gelatin (214). Application of the method is complicated by the presence of acids, bases, and salts, and by the effects of the solvents on the proteins studied.

Differences in electrophoretic mobility continue to be among the most useful methods of characterizing and separating the components of protein mixtures, and are referred to throughout this paper. The current methods have been reviewed (215) and a new apparatus for bulk fractionation has been described (216). A detailed interpretation



of the electroviscous effect in solutions of  $\beta$ -lactoglobulin has been offered (217).

*Solubility and thermodynamic activity.*—The detailed study of the solubility of  $\beta$ -lactoglobulin, begun some years ago by Sørensen & Palmer in the Carlsberg Laboratory, has been continued by Grönwald (218). The solubility is increased by the presence of glycine and glycine peptides, glutamic acid, and, most of all, by lysine and arginine. Egg albumin has the same effect, the solubility increasing in direct proportion to the concentration of the protein. Similar effects have been previously noted with other proteins and amino acids, and have been ascribed to the effect of the substances present on the dielectric constant of the solutions and to dipole-dipole interaction. Consequently, it is somewhat surprising to learn that the solubility of horse serum euglobulin is not increased by the presence of pseudoglobulin, which contributes a large dielectric increment to the solution, although it is increased by addition of serum albumin, the dielectric increment of which is much lower (219). The effect of pH on the solubility of both proteins is given, and the bearing of the phenomenon just described on the fractionation of the serum proteins is considered.

The solubility studies of Linderstrøm-Lang at Carlsberg long ago established that casein was a mixture of proteins. Ultracentrifugal studies at Upsala, and electrophoretic studies there and elsewhere confirmed this conclusion, and showed that the fractions hitherto obtained were mixtures also. The  $\alpha$  and  $\beta$  components (identified electrophoretically) have now been separated from one another by repeated fractionation with dilute acid and base (220). It is not claimed that the separation has eliminated other components from the fractions obtained.

Cellosolve-water mixtures of variable composition have been used to fractionate zein. Four initial fractions are described, but these can be fractionated further. Osmotic-pressures and optical-rotation measurements, and analyses of total nitrogen are used to resolve the total protein into three main components which differ in composition, molecular weight, and solubility behavior (221). Polyhydric alcohols are potentially useful protein solvents (222); two of them, ethylene glycol and glycerine, are already familiar.

A protein (gelatin) has once more been shown to increase the solubility of slightly soluble salts (223), and various amino acids have been shown to increase the solubility of euglobulin (224). A paper, which appears to leave out of account much recent work, reports that

ferrohemoglobin is without effect on the dielectric constant of aqueous solutions (225). The same paper considers the effect of the "ionic strength valence" of this protein on its behavior in solution, and comes to the not unexpected conclusion that the Debye-Hückel theory does not apply to dipolar ions. In a paper summarizing dielectric dispersion measurements on a number of proteins it is concluded that the dipole moments of a given protein may not only determine its activity coefficients in solutions of electrolytes, and its interaction with other dipolar ions, but may even determine the stability of the molecule in electrolyte-free solutions. Proteins having electrostatic dipole moments greater than 50 Debye units are denatured when their solutions are dialyzed (226).

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# THE CHEMISTRY OF THE NUCLEIC ACIDS AND NUCLEOPROTEINS

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This contribution makes no attempt to be a complete compendium of the literature but is designed as a critical survey of the present position in a few main sections of this field. Considerable difficulties and delays have been encountered in obtaining copies of certain journals and any omissions on this score are regretted.

## NUCLEOPROTEINS AND NUCLEIC ACIDS

*Isolation of nucleoproteins.*—Many methods for isolating nucleoproteins involve a stage, either during the extraction or more generally in the precipitation process, which is relatively drastic and may produce an alteration in the chemical and physical properties of the nucleoprotein; thus the majority of preparations involve precipitation of the extracted material with hydrochloric or acetic acids. The extraction processes vary considerably and employ water (1 to 13), dilute alkaline solution (14 to 17), sodium chloride solution (18, 19, 20), or buffer solutions of pH values ranging from 4 to 11 (21 to 26), followed in each case by precipitation with acid. It has been suggested (27) that nucleoproteins prepared thus are of variable composition, the precipitated nucleic acid carrying with it varying quantities of loosely bound protein, and these methods are now considered unsatisfactory (28) in view of the possible rupture of the nucleic acid-protein bond during the acid precipitation. A more controlled extraction of liver nucleoprotein is that in which the tissue was treated with a solution containing 0.03*M* sodium bicarbonate and 0.5*M* potassium chloride (29, 30) and the nucleoprotein precipitated by adjusting the solution to pH 4.2. Even these conditions may, however, be too drastic, and more recently attention has been concentrated on modifications of the original mild methods (1 to 11, 15), coupled with precipitation of the nucleoprotein with saturated ammonium sulphate (12, 31) or calcium chloride (6, 7, 32) solutions. Thus an early method (32) has been modified (33) whereby fresh pulped calf thymus is extracted with water at 5° C. for twenty-four to thirty-six hours, and after clarification of the extract the nucleoprotein is pre-

cipitated either by the addition of an equal volume of 2 per cent sodium chloride solution or of 0.2 per cent calcium chloride solution. A satisfactory, gentle procedure, apparently of fairly general application for the isolation of desoxypentose nucleoproteins (34 to 39), depends on the rather remarkable changes in solubility of the nucleoproteins in sodium chloride solutions of different strengths. They dissolve in 1*M* sodium chloride, forming viscous opalescent solutions, but are insoluble in 0.14*M* sodium chloride, although soluble in 0.02*M* or less or in pure water. No complete explanation of these phenomena has so far been put forward. The minced tissues, after being washed with 0.14*M* sodium chloride solution to remove cytoplasmic material, are extracted with 1*M* sodium chloride (2*M* in certain cases) and after clarification of the extract the nucleoprotein is precipitated in a distinctly fibrous form by dilution with sufficient water to bring the sodium chloride concentration to 0.14*M*. The nucleoprotein may also be precipitated from the concentrated salt solution by the addition of alcohol (13), as has been used for the preparation of nucleoproteins during the isolation of nucleic acids (40, 41); this method is, however, more likely than the former process to bring about denaturation of the protein. Both the desoxypentose nucleoprotein and the pentose nucleic acid have been isolated from the same tissue sample (42) by precipitating the aqueous extract of minced rat liver with 0.4 per cent calcium chloride, and extracting the desoxypentose nucleoprotein from the solid with 10 per cent or 1*M* sodium chloride. The pentose nucleic acid was extracted from the residue with boiling 10 per cent sodium chloride, a process which was not considered to change the composition of the pentose nucleoprotein (43).

The isolation of nucleoproteins from cells possessing a resistant wall has been satisfactorily accomplished (11, 28) by disintegration of the cell by means of intense audible sonic vibrations (44), the nucleoproteins being then extracted from the cellular debris with water and precipitated with 0.1*N* hydrochloric acid (11) or ammonium sulphate (28).

Development of the technique of differential centrifugation has provided an important method whereby the macromolecular nucleoproteins, generally in association with lipoid material, may be isolated from various tissues with a minimum of chemical action. This has permitted the preparation of the active nucleoprotein fraction of the Rous sarcoma I (45) and chicken tumour I (46, 47), and of various fractions from chick embryo (48, 49) and mammalian cells and tis-

sues (50, 51, 52). The method is also used extensively for the isolation of the virus nucleoproteins (53, 54, 55).

*Isolation of nucleic acids from nucleoproteins.*—Decomposition of a nucleoprotein into its constituent nucleic acid and protein is most easily brought about by alkaline hydrolysis (56), followed by removal of the protein with colloidal iron and precipitation of the nucleic acid in acid solution. An obvious objection, however, is that the alkalinity also causes some degradation of the nucleic acid. This degradation has no doubt often been considerable (57), but in some cases (see under viruses) this is apparently the only method which has so far been available; an alternative successful procedure is hydrolysis of the crude nucleoprotein with pepsin (15, 58, 59). A remarkably simple preparation of the desoxypentose nucleic acids from the nucleoproteins of calf thymus and rat liver is that in which the nucleoprotein solution in water or 1M sodium chloride is saturated with sodium chloride, the deposit of protein removed, and the nucleic acid, in a highly polymerised state, precipitated by the addition of alcohol (8, 42, 60, 61).

Complete separation of the protein and nucleic acid of nucleoproteins may be effected by decomposing the nucleoprotein in 0.5 per cent sodium carbonate solution at 50° C. for one to two hours and shaking the solution, neutralised to pH 7, with chloroform containing a small amount of a foam-preventing agent such as amyl alcohol; the protein concentrates at the interface forming a chloroform-protein gel which is easily separated by centrifugation (11). This method has yielded successfully the nucleic acids from the nucleoprotein of *Streptococcus pyogenes* (11) and from a fraction isolated from type III pneumococci (62), and the desoxypentose nucleic acid of thymus (34).

Procedures used in special cases but yet to be tested in wider fields are the heat-denaturation of tobacco mosaic virus nucleoprotein (63), the electrophoresis of tuberculin nucleoprotein (13), and dialysis of thymus nucleoprotein dissolved in 1M sodium chloride against 1M sodium chloride, which results in diffusion of the protein (34).

*Direct isolation of nucleic acid.*—It has repeatedly been stated that the methods usually adopted for the direct extraction of nucleic acids are unsatisfactory (64, 65, 66), and in spite of the fact that nucleic acids are labile towards alkali, the majority of the procedures employ sodium hydroxide. By use of this reagent nucleic acids have been obtained, for example, from yeast (58, 65, 66), pancreas (67) and

various animal tissues (68, 69), onion bulbs (70), malignant tissue (71, 72, 73), and bacteria (74 to 77). Extraction with sodium chloride solution yielded nucleic acid from yeast (18) and liver (41); in the latter case a nucleoprotein was probably isolated and subsequently converted into the nucleic acid during purification (see below).

In general, it would seem to be desirable to prepare nucleic acids by isolation and subsequent decomposition of the nucleoprotein.

*Separation of pentose and desoxypentose nucleic acids.*—It was early realised (78) that a tissue could yield both pentose and desoxypentose nucleic acids depending on the method of isolation, but the significance of the nature of the nucleic acid in this connection seems to have been lost sight of in subsequent work. The apparently contradictory statements that sodium chloride solution extracts a pentose nucleic acid from liver (41) and a desoxypentose nucleoprotein from thymus gland (34) emphasise this point. The fresh minced thymus glands were first washed with 0.14*M* sodium chloride solution and the desoxypentose nucleoprotein then extracted with a one molar solution. The dried liver powder on the other hand, was extracted directly with 10 per cent sodium chloride, and the "crude nucleic acid" purified first by precipitation with barium acetate and then with glacial acetic acid; both procedures have previously been stated to precipitate pentose nucleic acids, but not desoxypentose nucleic acids, and also to remove any protein impurity (40). For the isolation of either a pentose nucleic acid or a desoxypentose nucleic acid, it would therefore seem that two procedures are possible, (*a*) fractionation of the nucleic acids using barium acetate and glacial acetic acid as the precipitant, or (*b*) fractionation of the nucleoproteins by means of sodium chloride. The first method has been described in the case of the nucleic acids of pancreas, the pentose nucleic acid being precipitated with acetic acid and the desoxypentose nucleic acid with alcohol (79); the second method, as has been mentioned, was used for the nucleic acids of rat liver (42).

#### PROPERTIES OF NUCLEOPROTEINS

The nucleoprotein isolated by mild methods (33, 34) from calf thymus has a molecular weight of the order of  $2 \times 10^6$  (80) and its solutions in 1*M* sodium chloride show the high viscosity and marked streaming birefringence generally associated with highly asymmetric macromolecules. Once the nucleoprotein has been dissolved in water or in 0.02*M* sodium chloride solution, changes occur which result in

the nucleoprotein being less fibrous when reprecipitated in 0.14M salt (34), and its solutions in 1M sodium chloride are less viscous and show less streaming birefringence than those of the original nucleoprotein. These changes are apparently permanent and cannot be attributed to fractionation of the nucleoprotein. Viscosity measurements (33), however, are only in partial agreement with these observations; nucleoprotein extracted with water from calf thymus showed a much lower viscosity in dilute buffer (0.005M potassium acid phosphate plus 0.005M dibasic sodium phosphate) than in the same buffer containing 5 per cent sodium chloride, which observation would suggest that the viscosity change is reversible. A more detailed comparison of the two apparently conflicting observations cannot be made owing to the lack of experimental data recorded (34).

*Nature of the linkage between nucleic acid and protein.*—Until comparatively recently it was not infrequently assumed that the bonds between nucleic acid and protein are invariably electrovalent. This form of linkage may have been produced artificially by the relatively drastic methods of preparation of the materials examined. That supposition is supported by the results of a direct comparison of the nucleoproteins of streptococci (28), the nucleoproteins having been precipitated from the aqueous extracts by acid on the one hand and by ammonium sulphate on the other; in the latter case it is to be emphasized that the nucleoprotein had not come into contact with either acid or alkali at any stage. The examination of the nucleoproteins, which contained a pentose nucleic acid (11), was made by four methods, (a) deproteinisation by shaking with chloroform, (b) fractionation by ammonium sulphate, (c) precipitation with neutral calcium chloride solution, and (d) measurement of electrophoretic mobility. The natural nucleoproteins, unlike their acid-precipitated congeners, were completely soluble on the acid side of their isoelectric point. The most interesting results were observed in the precipitation by neutral calcium chloride; both the acid-treated nucleoprotein and an artificial protein nucleate included in the investigation formed an immediate precipitate, whereas no precipitation occurred with the natural nucleoprotein. These data imply either that the phosphoric acid groups of the natural nucleoprotein are bonded in such a manner as not to be available for reaction with calcium ions, or alternatively that the calcium salt of the natural nucleoprotein is soluble. Whatever the true explanation, it is clear that acid precipitation of the pentose nucleoproteins of streptococci does change their properties.

The nucleoproteins of cell nuclei (34), when extracted by mild methods, give evidence that the bond between the desoxypentose nucleic acid and the protein is electrovalent. Thus dialysis of a solution of the nucleoprotein of trout sperm in 1*M* sodium chloride against 1*M* salt solution resulted in the diffusion of the protein through the membrane, leaving the highly polymerised desoxypentose nucleic acid behind. Furthermore, extraction of the nucleoprotein solution with a chloroform-octyl alcohol mixture caused accumulation of the protein at the interface whilst the nucleic acid remained in solution (34). The desoxypentose nucleic acid and protein components of the nucleoprotein of tuberculin were separated by electrophoresis (13, 81) suggesting the presence of an electrovalent bond, but it should be pointed out that an acid precipitation was employed in the preparation. There are indications that the bonds in the pentose nucleoprotein of haemolytic streptococci are stronger than a dissociable salt linkage (25). The generalisation has been put forward (82) that the bonds between pentose nucleic acid and protein are covalent whereas those between desoxypentose nucleic acid and protein are electrovalent, but the evidence so far obtained with carefully prepared nucleoproteins is as yet insufficient to warrant this generalisation, which is certainly not in agreement with the data obtained from the virus nucleoproteins (see below).

The problem of the nucleic acid-protein bonds in nucleoproteins is complicated by experiments which suggest that there is a difference in properties when the cell nuclei of normal rat liver are isolated at pH 6.0 to 6.2 and at pH 3.8 to 4.0 (39). In the former case the nucleoprotein was easily extracted with 5 per cent sodium chloride solution, whereas in the latter little or no extraction occurred. Denaturation of the protein, which might conceivably have caused insolubility of the nucleoprotein in the sodium chloride solution, was not responsible for the firmly bound state, because, as was pointed out, this is found also in the nuclei of chicken erythrocytes prepared at pH 6.8 to 7.0, a treatment considered unlikely to cause denaturation. Further, denaturation of the protein is generally regarded as liberating the firmly bound nucleic acid from the protein, as for example in tobacco mosaic virus (63).

*Electrophoretic and other studies.*—The interaction of nucleic acids with various proteins to form complexes or protein nucleates has been known for some time (see for example 83, 84), but it is only recently that these complexes have been studied systematically

and their properties compared with those of nucleoproteins isolated from tissues. The method of investigation generally employed is that of determining the electrophoretic mobility, and it has been observed that some interaction occurs between thymus desoxypentose nucleic acid and serum albumen (85), although it could not be decided from the data obtained whether several firmly bound nucleoprotein compounds or only loose complexes were formed; the latter appeared more probable since it was observed that the electrolytic environment played an important part in determining the electrophoretic behaviour. Electrophoresis of mixtures of ovalbumen and yeast ribonucleic acid (86) gave similar results, clear evidence of complex formation being obtained in the isoelectric region of the ovalbumen, although in more alkaline solutions the two constituents migrated independently. Addition of the nucleic acid to ovalbumen in solutions more acid than the isoelectric point brought about partial precipitation of a complex.

It has not always been appreciated that resemblance of electrophoretic behaviour does not necessarily imply complete chemical similarity. This point is borne out by the electrophoretic comparison at pH 7 of (a) a nucleoprotein solution which is not precipitated by the addition of calcium chloride, and (b) a solution of the same nucleoprotein which had been treated with acid and could be precipitated by calcium chloride (28). The mobilities of the two specimens were very similar, that of the natural nucleoprotein being only slightly higher than that of the acid-treated sample, but no definite component split off during the electrophoresis of the latter material. It was concluded that both specimens were "nucleoprotein," but stress was laid on the excellence of the calcium chloride precipitation test for "showing changes that take place in nucleoproteins." Liver nucleoprotein has also been observed to migrate as a single component, although in this case the bond between nucleic acid and protein is such as to permit both components to be separated by dialysis (34). In contradistinction to these observations, the nucleoprotein of tuberculin (81) splits off a highly mobile component, largely nucleic acid, in solutions more alkaline than pH 5, although at pH 5 and pH 2.2 the nucleoprotein fraction traveled as a single component. This was held to show that in these solutions either the electrical properties of the nucleic acid and protein components were identical below pH 5, or a dissociation occurred at pH 5, releasing the nucleic acid. The latter explanation was preferred (81) and it was suggested that an association could occur between the imino group of histidine in the protein and a sec-



ondary phosphoryl group of nucleic acid, both of which dissociate in the region of pH 5 to 6. It was concluded (87), largely on the basis of an electrophoretic examination of the nucleoprotein of calf thymus between pH 3 and 9, that the nucleoproteins are definite compounds with a constant ratio of nucleic acid to protein, but the preceding observations suggest to the writers that this conclusion is not entirely justified. The electrophoretic evidence would only point to such a conclusion if the natural nucleoprotein and the artificial protein nucleate, prepared by mixing the unaltered nucleic acid and protein of the original nucleoprotein, were found to behave differently when examined electrophoretically, the latter splitting into two fractions at a certain pH value.

Addition of various albumens to solutions of sodium desoxypentose nucleate of thymus reduced the viscosity of the latter to values between that of the original nucleic acid solution and that of the protein (88), and at the same time diminished the streaming birefringence of the sodium nucleate solutions. The ability of the proteins to lower viscosity was dependent on their being in the native state since heat-denatured proteins were considerably less effective. These phenomena were at first attributed (88) to a polymerisation of the sodium nucleate although alternative explanations (89), such as complex formation between the protein and the nucleic acid, are feasible, and subsequently (90) it was considered that aggregation of the nucleate particles, leading to a more globular form, would account for the loss of streaming birefringence and structural viscosity.

Examination of solutions containing horse serum albumen and sodium thymus desoxypentose nucleate (90) showed that the osmotic pressures of the mixtures were in nearly every case almost identical with the value for the protein component, a result which was interpreted as suggesting that the presence of the protein causes aggregation of the nucleate ions and adsorption of the sodium ions onto the resulting micelle, thus rendering them osmotically inactive. Some preliminary experiments have been recorded on the adsorption of nucleate ions onto a protein monolayer (34) but no details were given.

*Virus nucleoproteins.*—The isolation and properties of virus nucleoproteins have been described elsewhere (53, 54, 55, 91, 92, 93) and this review is confined to the nature of the bonds between nucleic acid and protein, and to the distribution of the nucleic acid in the virus. All viruses so far isolated contain pentose nucleic acids, except those of psittacosis (94), vaccinia (95), rabbit papilloma (96), in-

fluenza A (PR8 strain) (97, 98), influenza B (Lee strain) (98, 99), and swine influenza (98, 100) which contain nucleic acids of the desoxy-pentose type. The nature of the bond between nucleic acid and protein is not known but seems to be relatively strong, except in the case of tobacco ring spot virus, of which part of the nucleic acid at least appeared to be less firmly bound (54), and of equine encephalomyelitis virus (96, 101), in which the linkage was reported to be weaker than in rabbit papilloma virus. The conditions necessary for decomposition of the viruses are, however, not the same. Thus, for example, only 62 per cent of the nucleic acid was liberated from bushy stunt virus (102) by treatment with 5 per cent sodium hydroxide for two hours at 4° C., whereas 68 to 78 per cent of the nucleic acid was liberated from tobacco mosaic virus when the 5 per cent sodium hydroxide was neutralised immediately after it had been added to the virus (103); the yield of this nucleic acid increased to 90 per cent if the virus was in contact with the alkali for two hours. Both the rabbit papilloma (96) and vaccinia (95) viruses were only decomposed by heating with 5 per cent sodium hydroxide for thirty minutes. An alternative procedure for decomposing tobacco mosaic virus is by heat-denaturation (63); this method is of importance as the nucleic acid isolated is very probably in, or almost in, its native condition.

The proteins of two strains of tobacco mosaic virus were electrophoretically homogeneous and identical (104), and a mixture of the nucleic acid-free proteins formed a single boundary although a mixture of the intact viruses gave a double boundary. From this it was concluded that both virus proteins were the same and that the difference in the two strains was due to the nucleic acid, as had been suggested previously (105) on the basis of the nucleic acid contents of the two strains. This interpretation, however, is faulty, since it has been shown that two different proteins can give a single boundary in an electrophoresis apparatus (106); furthermore, amino acid analysis showed that the difference in two strains of tobacco mosaic virus involves differences in the protein (107). This, however, does not exclude the possibility that differences may also occur in the nucleic acids. In agreement with this conclusion, the electrophoretic mobilities of these mutants of tobacco mosaic virus were not paralleled by the nucleic acid contents as determined by ultraviolet absorption spectra (108).

X-ray analysis (109, 110, 111) of tobacco mosaic virus did not indicate a concentration of the comparatively dense nucleic acid in any

particular part of the nucleoprotein, and it was suggested (104) that the virus may conceivably consist of either a long protein chain with nucleic acid side groups, or a regular arrangement of alternate nucleic acid and protein residues. The latter suggestion was preferred (104) on the basis of the improbably large molecular weight of the protein in the former scheme. In contrast with this conclusion, comparison of the molecular size of the tobacco mosaic virus protein and the nucleic acid obtained from it (63) led to the view that the nucleic acid exists as a thread-like macromolecule, of which the length is that of the intact virus. From measurements of absorption of polarised ultra-violet light by tobacco mosaic virus particles, oriented by streaming through a quartz capillary, it was considered probable that the pentose nucleic acid is arranged in an ordered manner and that the planes of the purine and probably pyrimidine rings are parallel to one another and perpendicular to the long axis of the molecule (112).

#### STRUCTURE OF NUCLEIC ACIDS AND THE TETRANUCLEOTIDE HYPOTHESIS

Originally the term "tetranucleotide" indicated the occurrence of the four appropriate nucleotides or the corresponding nitrogenous derivatives in equimolecular proportions in the decomposition products of a nucleic acid, and was thus used to describe certain of the then known nucleic acids at a period when these were regarded as having a molecule so simply composed. Now that the complex nature of nucleic acids as polynucleotides is established, the term is still applicable in this sense to such polynucleotides as conform to the tetranucleotide ratio in their nucleotide contents.

Later, as a development arising from the recognition of this complex character, the name has been used to denote a unit, consisting of one molecule of each of the four nucleotides, which by recurrent combination with itself forms the polynucleotide; it seems to be implied that the mode of this union is uniform throughout the polynucleotide. Finally, it has been postulated that in each of these units the four nucleotides are always combined in a fixed manner in an unchangeable sequence. As a logical outcome of these later hypotheses there has arisen the conception of certain polynucleotides as "polytetranucleotides."

The term "tetranucleotide" has thus a graded series of implications ranging from a statistical expression of analytical results to a definition of an exact chemical structure, and in order to avoid con-

fusion the sense in which the name is being used must be defined; the terms "statistical" and "structural" tetranucleotide are suggested for this purpose. It is opportune to review and assess the evidence on which these interpretations are based, all the more so because the importance of polynucleotides in the biological fields makes it essential that the possibilities of resemblance or divergence between polynucleotides from different sources should be clearly recognised so that conjectures involving nucleic acids may be based only on accepted chemical knowledge. The question of the existence of structural tetranucleotides has been discussed briefly (89, 113).

Present information allows polynucleotides to be classified in two main groups depending on the nature of the component sugars as pentose or desoxypentose. The existence of hybrid polynucleotides containing both types of sugar has been postulated (114), but it has been pointed out that in the absence of experimental evidence the suggestion is at present purely speculative (115). The literature also records the isolation of materials which on decomposition yielded the fragments of both types of acid, but it is probably correct to regard these as mixtures, in so far as their nucleic acid components are concerned, until the reverse has been proved. Where examination has been adequate, polynucleotides are characterised up-to-date by the presence of the nitrogenous radicals guanine, adenine, and cytosine, together with uracil in the pentose polynucleotides and thymine in the desoxypentose polynucleotides. Tuberculinic acid A (74, 116, 117, 118) seems so far to be unique in containing 5-methylcytosine (65, 119), but the presence there of this pyrimidine does suggest the need for close identification of pyrimidine components, perhaps more thorough than hitherto.

*Origins of the tetranucleotide hypothesis and the newer conception of molecular sizes.*—Although Miescher formed a picture of "nuclein" as a multi-basic, phosphorus-containing acid of molecular weight so large as to prevent dialysis, those properties were seldom stressed during the earlier part of the present century (60, 120, 121), but instead the development of the subject followed increasingly problems of detailed structure. This concentration of effort led to the wide assumption, chiefly from five causes (89), that the molecules of both types of nucleic acid, typified by the desoxypentose acid of thymus and the pentose acid of yeast, consisted of simple tetranucleotides. Recent data have shown that nucleic acids are considerably larger than tetranucleotides (for summaries and references, see 89, 122); the sizes

vary with the type and source of the acid and with the method of isolation and purification, but the general assumption of polynucleotide character for all nucleic acids seems to be justified. Nevertheless, the extent to which information obtained on isolated samples of polynucleotides can be applied to consideration of them in their native states is more obscure. Some methods employed in isolation may bring about considerable alteration in properties (59, 123), and although the suggestion (124) is probably correct that polynucleotides of lower size are formed by transverse breakage of larger molecules, the doubt remains that alterations of the polynucleotide may also be caused by processes of de- and repolymerisation, analogous to those observed in the case of thymus desoxypentose nucleic acid (61, 88, 125). Thus, measurements (126) of the effect of pH changes on the viscosities of solutions of a sodium desoxypentose nucleate of high molecular weight from thymus suggested that in neutral solution this salt acts as a linear polymer which is rapidly depolymerised to a considerable extent as the pH is altered from neutrality. The effect was complete at pH 2.6 and 11.6, and was reversed when these solutions were neutralised, the nucleic acid material then repolymerising slowly. Sedimentation velocity and diffusion measurements showed that both the degraded and the repolymerised polynucleotides have wider distributions of molecular weights than the original substance and that some of the molecules in the repolymerised acid are much larger than those present in the original solution. The sensitivity of these polynucleotide molecules to their concentration in solution, to the presence of added salts, and to changes of pH emphasises the difficulties inherent in studies of the structures of the acids in their natural condition.

The recognition of the complex character of nucleic acids undoubtedly opened afresh the whole question of their structure, but it is surprising to observe how readily there has occurred a mental superposition of the newer knowledge of molecular sizes onto the older ideas of the simple tetranucleotides, bringing with it the concept of the polytetranucleotide. Had the sizes of nucleic acid molecules been realised at an earlier date, it is doubtful whether the hypothesis of the structural tetranucleotide would have gained such a firm hold as is apparently the case. The relevant data are discussed below.

*Desoxypentose polynucleotides.*—Examination (124, 127) of the molecular sizes of samples of the polynucleotides from thymus prepared by different methods showed that treatment with alkali or enzymic preparations, or the action of heat, causes diminution as com-

pared with that of material obtained by a milder procedure (60), in which only neutral solutions and temperatures near 0° C. were used.

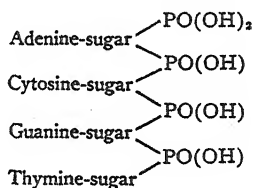
Enzymic degradation appears to proceed in two stages, possibly by two distinct enzymes, although the demarcation is indistinct and it is not clear whether the preparations causing the more deep-seated fission are not merely the more active. The  $\alpha$ -nucleic acid of thymus, the large polynucleotide of which the soluble salts show gel-forming properties, is converted by the nucleogelase of commercial pancreatin into the  $\beta$ -acid which no longer forms gels but is still precipitable by acids from solutions of its salts (120, 121). On the other hand, laboratory-made extracts of fresh or dried pancreas contain an enzyme, thymopolynucleotidase, which causes a more fundamental hydrolysis of the  $\alpha$ -acid, either previously isolated or while still present in the minced thymus gland (128, 129, 130); presumably it attacks the  $\beta$ -acid similarly. An enzyme causing similar effects has been recorded as present in a variety of animal and plant tissues and named thymonucleodepolymerase (131, 132).

The results of thymopolynucleotidase action were a fall in viscosity and rise in conductivity of the solution and the liberation of one acid equivalent (titrated to pH 9) for each four atoms of phosphorus present; the products were oligonucleotides corresponding to 3.9 nucleotides in size (129, 130). Oligonucleotides are amorphous powders and differ from the  $\alpha$ - and  $\beta$ -acids in being soluble in hydrochloric acid and in having sodium and magnesium salts which dissolve in their own weight of water to form solutions which are viscous but do not gel. They differ from nucleotides in being precipitated, as are the higher polynucleotides, by molybdate in acid solution. Estimations of guanine and adenine indicated that both purines were present in the proportions of one molecule for each four atoms of phosphorus.

Treatment of the  $\alpha$ -acid, free or in the thymus, with hot alkali produced a similar series of changes and resulted in mixtures of oligonucleotides and material resembling the  $\beta$ -acid; the size of the latter when prepared in this way was 8.5 to 10.5 (from the gland) or 18 to 19 (from the  $\alpha$ -acid) nucleotides, whereas the former corresponded to 3.3 to 3.6 and 3.2 to 4.0 nucleotides respectively. The materials prepared directly from the gland still contained at least 5 to 6 per cent of pentose-containing substances. Drastic alkaline extraction and purification of the desoxypentose nucleic acid of beef spleen (133) yielded material considered to be closely similar to the oligonucleotides. This product and the corresponding deaminated acid and thymic acid were

water-soluble penta-basic acids with approximately the correct analytical compositions and weights for molecules composed of four nucleotides (133, 134).

It has been postulated (113, 129, 133) that the oligonucleotides (I) are true "structural tetranucleotides" from which the polynucleotides are formed by polymerisation and that the tetranucleotide has the structure given to it by Levene (135). Definite positions were assigned (113) to the nucleotides of adenine and thymine, the relative orientation of the others being undetermined, on the basis of the following experiments. A mixture of mononucleotidase and oligonucleotidase (diesterase) from intestinal mucosa rapidly split off 25 per cent of the total phosphorus from the oligonucleotides. Further dephosphorylation occurred, and when this had reached 50 per cent the phosphorus-free fraction contained only one molecular proportion of nucleoside, consisting of a mixture of about equal parts of two nucleosides, those of adenine and thymine. Neither the guanine nucleoside nor mononucleotides were present in the solution.



I

If such postulates could be substantiated, an important advance would have been made. Whatever may come to light in the future, there is at present in the writers' opinion no evidence to justify the recognition of the existence of the "structural tetranucleotide," as will be seen from the following discussion.

First, only 65 to 70 per cent of the organically combined phosphorus was actually isolated as oligonucleotides in the alkaline or enzymic preparation, and there was thus ample possibility of the formation of other products. Second, the nature of the linkage attacked by thymopolynucleotidase is unknown; the enzyme is highly specific and does not attack yeast ribonucleic acid or a variety of representative potential substrates (128, 132). Third, the nature of the linkages attacked by alkali is unknown, and it is not clear whether these are the same as those split by the enzyme and thus whether the oligonucleotides

obtained by the two methods are the same or different. Fourth, all measurements made on the oligonucleotides, as on all polynucleotide material, give average results only. In a mixture of oligonucleotides, therefore, each molecule need not have the same composition or weight as its neighbours; it is stated (113) that fractional dialysis has so far failed to effect separation into fractions of lower and higher molecular weight, but it is doubtful whether the sensitivity of such a method would permit the recognition of small differences. A massing of any particular nucleotide in the polynucleotide chain is therefore not excluded. Fifth, even if the oligonucleotides were tetranucleotides, there is no reason why their nucleotides should be arranged uniformly in each molecule, and in the writers' opinion the experimental results could be explained in other ways which leave open the question of a regular sequence. There does not seem to be evidence that the adenine and thymine nucleosides both arise from the same oligonucleotide; the same statistical result would have ensued had each nucleoside been removed in its entirety from two different molecules. Furthermore, consideration and experience of enzyme experiments in this field suggest that their use in the interpretation of complex structures demands full information as to the constitutions of all the products and the specificity of the enzymes. In a fission of (I) to give the observed result the oligonucleotidase of intestinal mucosa must attack at approximately equal rates a 3- and a 5-phosphoester linkage. Knowledge whether the enzyme specificity discriminates between these is necessary for the interpretation of the results, but is unknown, since the positions of the phosphoryl groups in the desoxypentose nucleotides have not yet been determined; these were prepared by the action of the enzymes of intestinal mucosa on thymus desoxypentose nucleic acid (136, 137, 138). Sixth, Levene's structure (135) for a tetranucleotide cannot be accepted with certainty on the basis of the existing data. The proposal of (I) as the structure of desoxypentose nucleic acid (135, 139) was partly dependent on a supposed parallelism between that acid and yeast ribonucleic acid as regards the general form of their internucleotide linkages. The structure formerly proposed (140) for yeast ribonucleic acid has now been shown (141) to be at variance with the experimental facts in this respect (see below), and without drawing unwarranted parallels between these two acids it may eventuate that ideas on the internucleotide linkages of desoxypentose nucleic acids may also need revision.

On a wider issue the question arises whether desoxypentose nu-



cleic acids from different sources are one polynucleotide, uniform throughout nature, or whether there are a number of chemically distinct individuals. It seems frequently to be either assumed or implied that the former is the case (see however 142). The writers are not aware of facts which favour either alternative, but attention is drawn to the considerable possibilities of variation in structure of polynucleotides which could arise from divergences of molecular size, relative orientation and proportions of nucleotides, and nature of the sugar components.

Several instances are recorded (e.g., 68, 143) in which hydrolysis of nucleic acids from different sources yielded the nitrogenous bases in amounts differing considerably from those expected on the basis of the tetranucleotide ratio. Such results, however, need to be confirmed or refuted through improved technique, or other methods, and it may be that a beginning has been made (82) in the use of the diphenylamine colour reaction (144, 145) to determine the ratio of total purine to total pyrimidine nucleotides. The method does not distinguish between individual purine or pyrimidine nucleotides. The interpretation of even this simple estimation may be complicated, since it is stated on the one hand to be positive specifically for the sugar of the purine nucleotides (82, 146) and on the other to be given by all the sugar of a desoxypentose nucleic acid (147, 148); these divergences may depend on the exact conditions since the glycosidic linkages vary in stability. According to a recent report (82) all the desoxypentose nucleic acids so far examined, but from sources as yet unnamed, agree in having equimolecular proportions of total purine and total pyrimidine nucleotides.

It seems to be widely accepted that the sugar (thyminose) is *d*-2-desoxyribofuranose in all nucleotides. This may be the case, but it should be realised that the assumption of the furanose structure is based solely on the demonstration of its presence in the thymine nucleoside (149, 150, 151), and that the identification of the sugar itself applies only to the guanine nucleoside of the acid from thymus (152, 153, 154); the sugars of the other nucleosides (135, 155) and of nucleic acids from other sources do not seem to have been prepared. A detailed study of the identification and estimation of the sugars in several nucleic acids (147) by the Dische carbazole colour reaction assumes that the sugar is all desoxyribose, although authentic samples of desoxyribose and its guanine nucleoside gave inferior colours. Not infrequently the classification of a nucleic acid as "desoxyribose" nu-

cleic acid rests merely on a positive result in the Feulgen (156), Dische diphenylamine (81, 144), Thomas (157), or Kiliani (158) colour reactions; these are commonly regarded as specific for thymine, whereas in reality they demonstrate the presence of a desoxy-sugar, and according to the experimental conditions (see above) may refer only to the purine nucleotides.

*Yeast ribonucleic acid.*—This is the only pentose nucleic acid which has been considered seriously from the standpoint of a "structural tetranucleotide." The constitutions (159) and syntheses (89) of the component nucleotides have been reviewed, and since purines and pyrimidines are present in approximately the tetranucleotide ratio (160 to 165), the acid is a "statistical tetranucleotide." The original conclusion (56) that the sugar radicals are *d*(—)-ribose has been confirmed (166, 167, 168), at any rate as regards some, by identification as the ribonic phenylhydrazide and benziminazole derivative. Small quantities of *l*-lyxose benziminazole were also isolated, together with some *d*-arabobenziminazole; the formation of the latter has been observed as the result of epimerisation during the alkaline oxidation of ribose (169), but this may not explain its presence in products formed from the nucleic acid (168), and the possible presence of sugars other than ribose in nucleic acids should remain open for the present. In this connection it may be noted that the colours developed by ribose, arabinose, and lyxose in the Dische carbazole reaction for pentoses were so similar in intensity that these sugars could not be distinguished (147). Not infrequently a nucleic acid is classified as a "ribose" nucleic acid (e.g., 170, 171) solely by a negative colour reaction for desoxypentose and a positive result in one of the methods for detection or estimation of pentoses, which depend on the formation of furfural. Theories of the biogenesis of ribose have been reviewed (89).

As with the desoxypentose nucleic acids, alkaline and enzymic fission reduce the molecular size of yeast ribopolynucleotide without dephosphorylation. Milder conditions of hydrolysis than those causing complete fission into nucleotides converted an already partially degraded acid into a product considered to be a structural tetranucleotide (134). This and the corresponding deaminated material were water-soluble, approximately penta-basic acids of the correct analytical compositions and molecular weights of 1177 and 1121 (calculated 1304 and 1307). It has been pointed out (141) that these materials cannot have been true tetranucleotides; the deaminated tetranucleotide should have been hexa-basic since the hydroxyl of xanthine titrates

over the range pH 6 to 8 and its presence was demonstrated in deaminated yeast ribonucleic acid by electrometric titration. Moreover the low molecular size of the product was not confirmed (172), and it was concluded (141) that these fission products were really mixtures of small polynucleotides and that there is no evidence on these grounds for the existence of a "structural tetranucleotide."

At one time it seemed possible that a study of the enzyme ribonuclease (ribonucleinase, ribonucleodepolymerase) (see 122, 173) might provide evidence of a "structural tetranucleotide." Further data, however, show that the enzyme causes deeper fission; the average size of the dialysable products of the action of preparations of heat-stable pancreatic enzymes corresponded to that of a dinucleotide (128), and nucleotides were isolated from the products of digestion by crystalline ribonuclease (174). Nevertheless, the exact effect of heat-stable pancreatic enzymes is not clear; enzymes isolated from whole pancreas provide small and variable yields of the four nucleotides, whereas the claims that depolymerisation took place without formation of appreciable quantities of nucleotides arose from experiments with enzyme preparations made from commercial pancreatin. The possible parallel with somewhat analogous observations in the fission of thymus desoxypentose polynucleotide with pancreatic enzymes is worthy of note.

The results, on which exact positions of certain nucleotides relative to each other in a structural tetranucleotide were defined from enzyme experiments (175) or aqueous pyridine hydrolyses (176), seem to the writers to indicate merely that certain linkages in the polynucleotide are more labile than others. It is thus clear that there is at present no evidence for the "structural tetranucleotide" as a unit of yeast ribonucleic acid.

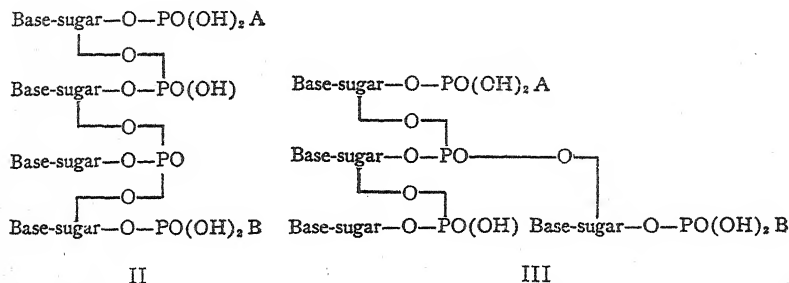
It is generally agreed that the action of the heat-stable pancreas enzymes renders about half of the phosphorus of the acid non-precipitable by the uranyl-trichloroacetic acid reagent (177) although still organically combined; the remainder resembles the original acid in being precipitated by the reagent and also, although less readily (178), by hydrochloric acid. It should not be automatically inferred that the precipitable material is unchanged acid. It has been pointed out (122) that yeast ribonucleic acid must contain at least two different types of linkage, one labile, the other resistant, to the action of ribonuclease, and several alternatives are possible, in that the liberated nucleotides may not come from every nucleic acid molecule, but may result from

transverse fission of the polynucleotide chain, or may be lopped from a main polynucleotide trunk bearing nucleotides as branches (141) (see below). In addition, hints are not lacking that all samples of the acid are not identical in structure; lability to alkaline hydrolysis is a variable property (179, 180), ribonuclease activity alters considerably with the sample of nucleic acid and is also sensitive to the age of the substrate solution (181), and only certain preparations of the acid give guanine-uridylic acid on hydrolysis (176, 182, 183).

New possibilities have been brought to light for the internucleotide linkages of yeast ribonucleic acid. In view of the complete agreement that the polynucleotide exhibits four phosphoryl dissociations and since hydrolysis results in the appearance of secondary phosphoryl groups, it might be assumed that the polynucleotide is a polymer of the structural tetranucleotide proposed by Levene (140) and that in consequence each of its phosphoryl groups, except the terminal one, should exhibit one primary dissociation. This view seems to be incompatible with the experimental facts (141). Samples of the acid, and of the deaminated acid prepared by a method which avoided any fall in molecular weight, were titrated electrometrically between pH 2 and 12. Comparison of the experimental curves with those constructed theoretically showed that for each four atoms of phosphorus there were three primary and one secondary phosphoryl dissociations. A hypothetical structural tetranucleotide forms a useful standard for the assessment of results (89), and if these observations are considered on the basis of a polytetranucleotide from this standpoint only, each individual unit of the polymer would contain one triply esterified phosphoryl group; the shape of the titration curves agrees best, but not absolutely, with a formulation in which this is the group of uridylic acid. Two dissociations would be primary and associated with doubly esterified phosphoryls, and one phosphoryl group would be singly linked to a sugar radical and would exhibit both primary and secondary dissociations. Possible formulae in agreement with the facts, including the presence of titratable, and hence unsubstituted, purine and pyrimidine hydroxyls, are II and III; polymerisation would occur either through the group A or B, the other member of the pair remaining singly linked.

Without necessarily assuming a uniform tetranucleotide, it was considered that structural relationships of this nature may occur in the polynucleotide, and three points are noteworthy. First, the literature shows a marked tendency for the phosphorus analyses to be low. In a

polymer of nucleotides all united successively through their phosphoryl groups, a deficiency of phosphorus could only occur by removal of the singly linked terminal phosphoryl, but the extent of the deficiency was often greater than could be accounted for in this way. In a polymer



of II or III it would be possible for up to 25 per cent of the total phosphorus to be absent without upsetting the main polynucleotide structure. Second, a structure can be visualised in which nucleotides form side chains and could be removed by the action of ribonuclease, leaving a molecule sufficiently large to resemble the original acid in its failure to dialyse and in its precipitation reactions. Third, mixtures of enzyme preparations containing a phosphomonoesterase and a phosphodiesterase produced only 75 per cent dephosphorylation of yeast ribonucleic acid (184), and these mixed enzymes caused only 75 per cent dephosphorylation after pretreatment of the acid with a boiled extract of pancreatin (185); these results are in agreement with the presence of one triply linked phosphoryl for each four atoms of phosphorus.

Russell's viper venom, containing a phosphodiesterase (186, 187) but only very weak nonspecific phosphomonoesterase activity, dephosphorylated yeast ribonucleic acid ultimately to 50 to 55 per cent (188). If during their action the enzymes were inactivated with cyanide ions, dephosphorylation continued slowly in the alkaline medium or more rapidly in 1 per cent sodium hydroxide, thus demonstrating the existence in the acid of alkali-labile phosphoryl linkages. These labile groups were assigned tentatively to the hydroxyls at C<sub>2</sub> of the sugar radicals, and a working hypothesis was suggested that the polynucleotides of intermediate size which do not suffer loss in molecular weight when deaminated, and hence are free from phospho-amide groups (113, 182, 183), may be composed of nucleotides joined mutu-

ally through phosphoryl at  $C_2$  and  $C_3$  of the pentose. Union of such polynucleotides through phospho-amide groups, e.g., guanine-uridylic acid, might form a large polynucleotide in its native state, and the ultimate presence or absence of alkali-labile phospho-amide groups in a given sample of yeast ribonucleic acid might depend on their survival or destruction in the alkali at present used to extract the acid from yeast.

*Other pentose polynucleotides.*—Cold alkali converted the higher polynucleotides of tobacco mosaic virus into particles of molecular weight of 15,000, and it was specifically stated that there is at present no evidence that a unit smaller than this but larger than a nucleotide exists as a fundamental unit of the virus acid (63). Alkaline fission yielded an insoluble trisodium guanylate apparently identical with that from yeast ribonucleic acid, and a uracil nucleotide isomeric with uridylic acid; the purine content was 20 per cent lower than that required by the tetranucleotide ratio (103).

A pentose nucleic acid (41), believed to occur in the cytoplasm, was isolated from sheep liver; part of the sugar was identified as *d*-ribose and the amounts of total purine and easily hydrolysable phosphorus were consistent with the tetranucleotide ratio.

The pentose polynucleotide of pancreas (26, 189, 190) contains the four nucleotides (67, 191, 192) in the ratio, it has been proposed, of one molecule of adenylic acid and each pyrimidine nucleotide to varying proportions of guanylic acid, ranging from two to four molecules (40, 43, 79, 193). The pentose nucleic acid of rat liver appears to resemble pancreas pentose nucleic acid in its high nitrogen:phosphorus ratio, which exceeds that required by a tetranucleotide (42).

No conclusions can be drawn as to the structure of pentose nucleic acids which have been characterised only by detection of some or all of the nitrogenous components, or by the demonstration of the pentose nature of the sugar (for example, 47, 76, 170, 171, 194 to 197) or by the isolation of incompletely identified nucleotides (198).

In view of the wider possibilities of isomerism of nucleic acids recognised now as compared with formerly, claims that two acids are identical, as for example yeast ribonucleic acid and triticonucleic acid of wheat germ (58), require confirmation.

*Terminology of nucleic acids.*—Some discussion has taken place on this subject. On the one hand (199, 200), it was proposed that the name chromonucleic acid should be used instead of thymus nucleic acid as the biological term for the substance described chemically as

desoxyribose nucleic acid, and that plasmonucleic acid should be substituted for yeast nucleic acid as the biological equivalent of the chemical term ribose nucleic acid. On the other hand (159, 201), the necessity was stressed in the light of present information of defining a nucleic acid by referring both to its origin and its type (pentose or desoxypentose). This would avoid confusion until such time as it can be stated with certainty either that two (or more) individual nucleic acids exist and that all examples of each are identical in chemical constitution, or that two (or more) types of nucleic acid exist and that each type comprises a number of examples of related but different constitutions; closer specification of the type would be permissible when justified on chemical grounds. The suggestion (123) has also been made that in the characterisation of different nucleic acids the treatment in the course of isolation, such as the degree of tissue autolysis, reagents used, etc., as well as the chemical and physical properties, should be described.

#### LOCATION OF NUCLEIC ACIDS IN CELLS

In an extensive survey of the nucleic acids of embryonic and adult tissues (202), the dried powders, freed from acid-soluble and lipid phosphorus compounds, were extracted with 10 per cent sodium chloride and the total nucleic acids precipitated with lanthanum acetate. Analyses for phosphorus, pentose, and desoxypentose confirmed the belief (202) that pentose nucleic acids are of general occurrence in animal cells, and indicated that rapidly growing tissues are characterised by high concentrations of both types of acid.

In the light of recent reviews (82, 122, 203), the remainder of this section is restricted to surveys of the criticism levelled at the widely accepted theory of the part played by nucleic acids in the nucleus and to the progress made in elucidating the mode of occurrence of cytoplasmic nucleic acids.

*Nucleic acids of the nucleus.*—For some time it has been considered as established that desoxypentose nucleic acids are located principally in the nucleus and that pentose nucleic acids characterise the cytoplasm. This concept is largely founded on the cytological application of ultraviolet photomicrography to demonstrate the presence of purine and pyrimidine rings and on the use of Feulgen staining to detect desoxypentose nucleic acids (for representative papers, see 204 to 209, and for reviews, see 82, 203, 210, 211). The hypothesis that nucleic acids play an essential part in the reproductive cycle of the cell and in

the genic perpetuation of inherited characteristics (212) is largely dependent on results achieved by means of these techniques, and hence the specificity of the Feulgen reaction in denoting the site of desoxypentose nucleic acids is a vital factor. Question of its validity might necessitate reconsideration of a large mass of conjecture and experimental conclusions. Criticism has been made and refuted (213 to 216), and it has been pointed out that the reaction is specific for desoxypentose only in the absence of lipoids (96). Serious doubts have now been thrown on its reliability as an indicator both of the total amount of desoxypentose polynucleotide material (113) and also of the location of such substances in the cell (113, 217).

Cellular nuclei contain, besides nucleic acid and histone, an acidic protein, chromosomin (217), postulated as the characteristic material of chromatin. It may be identical with, or related to, a new protein of cellular nuclei, reported earlier by other workers (57). Chromosomin has the property of taking up the basic, water-soluble dye produced by the interaction of decolorised magenta and hydrolysed desoxypentose nucleic acid, and hence the appearance of this dye in the nucleus of the cell does not, it was maintained, necessarily indicate the presence there of the desoxypentose nucleic acid. Furthermore, it was possible to stain chromosomes directly with the "developed nucleal stain" (218). It was suggested that desoxypentose nucleic acid is present in the nuclear sap, rather than in the chromosomes, and may form the mitotic spindle, and that chromosomin is "the chemical basis of inheritance" (219). Histone, on the other hand, was regarded as filling the role of regulating mitosis (220). Previously, desoxypentose nucleic acids were regarded as essential to the duplication of the chromosomes (211), and although no concrete proposals were made, there was a tentative assumption that the genes consist of desoxypentose nucleic acids combined with protein (210).

These postulates as to the part played by chromosomin and the location of desoxypentose nucleic acids outside the chromosomes have been adversely criticised (221, 222). Enzymic destruction of nucleic acid in the nucleus rendered the chromosomes not stainable by Feulgen's method, whereas hydrolysis of the protein component by trypsin did not alter the selective ultraviolet absorption of the nucleus in the region characteristic of nucleic acids. The parts of the chromosomes which can be stained by the Feulgen technique contained a high proportion of phosphorus, in accord with the presence there of nucleic acid, and isolated chromosome threads were found to contain 40 per



cent of desoxypentose nucleic acid (for references see 221, 222). Analyses of different tissues (220), which contain varying quantities of nuclear sap but show little variation in the amounts of nucleic acid, were quoted in opposition to the hypothesis that the nucleic acid is present in the nuclear sap. The fact that mitotic spindle fibres are positively birefringent with respect to their length, whereas desoxypentose nucleic acid is negatively birefringent, was held to be in apparent opposition to the suggestion that the spindle consists of a gel of desoxypentose nucleic acids (222). It was also stated (223) that the assumption of chromosomin as an integral constituent of the nucleus in no way affects the views which, chiefly with the aid of ultraviolet absorption measurements, have been advanced in regard to the protein and nucleic acid metabolism and the distribution of those substances in the nucleus. Replies to these criticisms have been made (219, 224). In view of this controversy, many may hesitate to acknowledge as yet definite functions or dominant roles for these cellular constituents.

*Nucleic acids of the cytoplasm.*—Comparatively little attention has been paid to the organisation of cytoplasmic nucleic acids until recent years. By high-speed centrifugation, fractions consisting of submicroscopic particles were obtained from chicken tumour I agent (46) and normal chicken embryo (48); these were concentrated separately from the nuclear material and toward the centripetal end. The particles from the tumour agent had high tumour-producing activity whereas those from normal embryo were inactive, and both contained lipid together with a pentose nucleoprotein. From the tumour agent, a pentose nucleic acid was isolated, having a broad absorption band with a maximum at rather shorter wave-length than 2600 Å; this was regarded as indicating the presence of a high proportion of guanine, and guanine nucleotide was isolated after alkaline hydrolysis (47, 50, 225). These observations constitute a marked advance in the study of the chicken tumour and in knowledge of the location of pentose nucleic acids in cytoplasm. Similar particles (microsomes), obtained from various tissues, had a constant composition irrespective of the source (51, 52), consisting of phospholipids and pentose nucleoproteins associated in definite proportions. Nucleoproteins have been found in conjunction with lipid material by other workers (for summary, see above and 203). The part played by microsomes in the structure of the cytoplasm has been discussed (52) but no fully substantiated proposal has yet been put forward. They have been regarded as fragmented mitochondria (50) and as independent cellular structures associated with

differentiation of the cell, and from which, for instance, the secretory granules of the pancreas are derived.

An interesting aspect of the nucleic acids of bacterial cells has been revived recently. For some time it has been known that Gram-positive pneumococci can become Gram-negative, and this change was brought about by extracting the cells in neutral solution (20) and by an enzyme, apparently identical with the pancreas enzyme which acts upon yeast ribonucleic acid (226). The material released into the solution during the former process contained pentose nucleic acid and a nucleoprotein (20, 227). A similar change has now been effected in yeast cells and Gram-positive bacteria by extraction with a solution of a bile salt (228), and it was also possible to restore the Gram-positive reaction by replacing the responsible material, an essential component of which appears to be the magnesium salt of a pentose nucleic acid; other salts of nucleic acid could not be plated back in this way. In agreement with the previous workers, the stainable material could be progressively extracted, that part on the surface of the cell being removed first.

#### INDUCED TRANSFORMATION OF PNEUMOCOCCAL TYPES

Among micro-organisms, the most striking example of the reproducible and controllable induction of inheritable and specific alterations in cell structure and function is the transformation of specific types of pneumococcus. This type of change has been brought about both *in vivo* and *in vitro*, and analogous transformations have been carried out in the field of viruses (for references, see 62). Avery, MacLeod & McCarty (62) have now isolated from type III pneumococci a biologically active fraction which in exceedingly minute amounts is capable under appropriate conditions of inducing the transformation of unencapsulated R variants of pneumococcus type II into fully encapsulated S cells of type III. Other variants are not transformed in this way. Examination of the active extract indicated, within the limits of the methods employed, that protein, unbound lipid, and serologically active polysaccharide were absent, and that it consisted principally, if not solely, of a sodium salt, in homogeneous viscous form, of a desoxypentose nucleic acid of molecular weight of the order of 500,000. It is possible, as the authors suggest, that the biological activity of the material is not an inherent property of the nucleic acid but is due to minute amounts of some other substance so intimately associated with it as to escape detection. If, however, as

the evidence strongly suggests, the transforming principle is a sodium salt of a desoxypentose nucleic acid, this type of polynucleotide must be regarded not merely as structurally important but as functionally active in determining the biochemical activities and specific characteristics of pneumococcal cells. This would appear to be the first occasion on which specific transformation has been experimentally induced *in vitro* by a chemically defined substance, and its implications are of the greatest importance in the fields of genetics, virology, and cancer research.

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## X-RAY STUDIES ON COMPOUNDS OF BIOCHEMICAL INTEREST

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Every three years, a review paper on this subject has appeared in the *Annual Review of Biochemistry*. These papers have thus adequately reviewed the field until the end of 1941. Since then, while enough papers have appeared to warrant a review, the war has, nevertheless, sharply reduced the published research. The reviewer believes, therefore, that he may justifiably devote part of his allotted space to an analysis of the general approach of x-ray methods to the study of materials of biochemical interest.

All x-ray scattering experiments are similar in principle. A well defined beam of monochromatic or nearly monochromatic x-rays is allowed to fall on the specimen. This radiation is scattered in all directions and is recorded by means of photographic film. The pattern of scattered radiation is determined by the geometry of the experimental set-up and by the scattering specimen. Because it is determined by the structure of the specimen, one attempts to set up a one-to-one correspondence between pattern and specimen structure. However, while one can determine the pattern of scattered x-rays if the structure is known, the reverse proposition is not true, and only sometimes (rarely for the complex structures that interest us here) can the structure of the specimen be determined from the x-ray scattering. Nevertheless, much useful information is obtainable from the x-ray diagrams. The widest use is for purposes of identification. Here identification is used in its broader sense—referring not only to identification of materials but also to tracing of chemical and physical changes. Heretofore, the powder (Debye-Scherrer) method has been used almost exclusively for such purposes. This method is satisfactory for comparatively simple materials. However, when applied to complex biological materials it is far from satisfactory, for often the deceptively simple x-ray diagrams, e.g., as obtained with proteins, are far from adequate to characterize the complex structures responsible for them.

The general problem will be considered from three different view-

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points, each one of which is broadly divisible into two parts. First, the purpose of the x-ray experiment will be discussed. The experiment may be planned either to identify a material (in the broader sense) or to answer one or more clearly defined chemical or physical questions. Secondly, a division is possible on the basis of orientation. The specimen may possess some specific direction or directions of orientation or be free from any orientation. Lastly, a natural division occurs on the basis of angle of scattering. Until recently this division could not have been made, for most x-ray work has been what may be called wide angle scattering. Within the last ten years an increased interest has been manifest in the scattering at small angles. This low angle scattering is of potentially great interest to workers in the biological fields.

*Purpose.*—In most non-biological x-ray studies, by far the greatest proportion of the work has been done using the powder method (non-oriented specimen) for identification purposes. Even in the study of biological materials a large part of the studies to date have been of this character. There is considerable doubt in the reviewer's mind as to the value of such studies. Biological materials are generally complex structures of comparatively large dimensions. When the powder diagrams themselves are well defined and adequately complex, then perhaps the powder method can and should be used for the study of complex structures. Generally such definition and complexity are not found in the powder diagrams. For example, even crystalline proteins give rather poor powder diagrams. In such cases, it should be realized that the x-ray evidence is merely suggestive but is not conclusive.

To settle questions of identity, such as the possible reversal of protein denaturation, either more complex diagrams must be obtained by the use of higher orientation in the specimen, or by improved experimental technique. Otherwise, the x-ray method simply must be considered as giving an equivocal answer.

X-ray diffraction methods can be used for purposes other than identification. When Friedrich & Knipping (1) in 1912 did their pioneer experiment in x-ray diffraction both the crystal and the x-ray spectrum they used were unknown quantities. The pioneer work of the Braggs' (2, 2a) produced the correct structure for sodium chloride, and this was the Rosetta stone for the quantitative study of x-ray spectra. The x-ray crystallographer could use known x-ray wave lengths to work out the structures of other crystals. To start with,

crystals were almost always chosen of materials whose chemistry was well established and gradually the goal of the x-ray worker became the determination of the structure of crystals. A structure, once established, usually did not revise or increase our chemical knowledge although gradually with the increasing number of completely worked out structures a new branch of chemistry, crystal chemistry, evolved. This dealt with bond lengths, bond angles, coordination numbers, etc. When one studied a crystal, it was with the aim of working out its structure; to stop before one had located substantially all the atoms (excluding hydrogen) was to acknowledge defeat, for the structure was the goal. Indeed in most cases, the researcher was well pleased when the structure turned out to agree with the known chemistry for then he was probably right! There were notable exceptions to this sort of approach fortunately, particularly in the schools created by the Braggs and by Bernal. These workers did x-ray work which led to important revisions in chemical knowledge. Bragg (3), for example, literally rewrote silicate chemistry as a consequence of his x-ray studies. There is an important difference, however, between his approach and that of Bernal. Bragg studied materials whose chemistry was obscure and revised that chemistry on the basis of complete structure determinations. Bernal, on the other hand, was one of the first to see clearly that incomplete x-ray work (incomplete in the sense that no attempt need be made to work out a complete structure) could often answer important questions. The classic example of this sort of work is the research of Bernal and co-workers on the sterols (4, 5, 6). He clearly showed by comparatively simple single crystal studies that the Windaus formula for the sterol skeleton could not be correct and gave strong support for the now accepted structure.

Such incomplete studies can be useful because of a rather interesting characteristic of the analysis of x-ray data. All x-ray diagrams consist of lines or spots of varying intensity. The first part of the analysis is geometrical; one makes computations based on the locations of the lines or spots. Only after the geometrical analysis is complete is an attempt made to use the intensities of the reflections. There are many stages in both the geometrical and the intensity analyses, and one obtains at each step results which can often be useful. Thus an unequivocal determination of the dimensions of the unit cell combined with a density measurement permits the computation of a molecular weight or at the worst a simple multiple of it. Similarly the unit cell size may place limitations on the dimensions of a molecule. Thus for

biotin (7), x-ray work in a non-destructive way not only gave a value for the molecular weight but also indicated that the molecule had to be fairly flat. In biochemical work, one often has very little of a given material. It may be very important to be able to get, in a non-destructive way, a value of the molecular weight and some idea of molecular size, shape, and perhaps symmetry. It should be emphasized that only rarely can such information be obtained from powder diagrams. Almost always experiments with oriented specimens are required.

Of course, a complete structure determination is the highest aim of an x-ray investigation. When this aim is attained, then all that x-rays can tell about the chemistry of the material is revealed. Unfortunately, the determination of structures is based essentially on an analysis of the intensities of the individual reflections. This analysis is not straightforward and only rarely can it be carried through in the case of complex structures.

*Orientation.*—When a single crystal is rotated in a monochromatic x-ray beam, it moves through a number of critical positions in which Bragg's law is obeyed by different sets of lattice planes. At the moment at which Bragg's law is obeyed, the lattice plane will reflect the beam as does a mirror. As the size of the unit cell increases, the number of reflections which occur for a given motion of the crystal also increases. Inasmuch as most structures of biochemical interest consist of large molecules and therefore large unit cells, we must expect to find a large number of reflections in their x-ray diffraction diagrams. In a single crystal x-ray experiment, each reflection has a particular direction in space. When the specimen has no orientation (for example, is a randomly oriented mass of small crystals), each single crystal reflection is spread out into a right circular cone of radiation whose axis is the x-ray beam. The intersections of the cones with the photographic films are the Debye-Scherrer lines. For large unit cells, most of the cones will be composite in origin, i.e., will be due to many different sets of lattice planes. Moreover, only the strongest of the possible reflections will generally be observed. Reflections clearly visible in single crystal diagrams will often be unobservable in the powder diagram. Thus powder diagrams of complex materials can be comparatively simple in appearance, deceptively so, and hence cannot adequately characterize a complex structure.

Of course, the powder method has its advantages. Many materials are readily obtainable only in the form of a fine powder of very small crystals. The actual x-ray experiment also is very simple—so also

are the measurement of the films and the computations which can be made from the measurements. Nevertheless, the positive results that can be obtained by the powder technique when applied to the large structures of biological interest are so meager that the reviewer feels that most strenuous efforts should always be made either to impose as high an orientation as possible on the specimen or to obtain a single crystal large enough to work with.

Molecules of biochemical interest are often quite anisotropic in shape and therefore often form equally anisotropic crystals. A variety of ways have been used to introduce orientation into masses of such molecules. Velick (8) recently studied oriented smears of tuberculostearic acid. Many workers (9, 10) have drawn oriented fibers from non-oriented starting masses. Astbury and others (11, 12, 13) have even produced secondary orientation, i.e., orientation in two directions by combined drawing and rolling procedures.

Much more single crystal work would also be done if it were generally realized that one can work with comparatively small crystals. It is, for example, possible to mount and study small needles, 0.1 by .01 by .01 mm in size. Some experience and much patience are needed when the crystals are very small but such work can be done (7) and, because so often it is the only way to obtain useful x-ray data, should be done.

*Angle of scattering.*—In the earlier x-ray diffraction studies, the significant scattered radiation occurred at angles of five degrees or more. However, the larger the molecules (and therefore the periodicities involved) the smaller the minimum angle at which significant scattering appears. Thus, for sterols, reflections from lattice planes occur within two degrees of the central beam (5), and for many crystalline proteins, the angle of deviation of some of the reflected rays is less than one degree (14). More recent work on viruses (15) and on collagen (16) required the study of the scattered radiation within a few minutes of the incident beam direction. A natural division thus exists based on the angle of scattering. The author will not, however, name some angle of deviation as a dividing point, but will base the division on the origin of the scattered radiation. The patterns often are comparatively independent of one another; thus, the wide angle diagram of a protein may be insensitive to the action of swelling agents while the low angle pattern may be very sensitive.

The origin of the wide angle diagram has been so thoroughly discussed in the past that it will not be considered here. The low angle

scattering will be discussed in some detail for its character and utility are not too widely known nor are its theory and technique in anything like a highly developed state.

The reviewer has had occasion to discuss low angle scattering recently in a paper on high polymers (17). Low angle scattering can exhibit either more or less distinct maxima (15, 16), or a continuous type of scattering which increases in intensity as the angle of scattering decreases (18, 19). Sometimes both types are found simultaneously. The maxima apparently arise for many reasons, some of which are clearly understood and others which are not. In the case of bushy stunt virus, the lines originate in a large scale, three-dimensional, crystalline arrangement of the virus molecules (20), while in tobacco mosaic virus a two-dimensional, liquid crystalline arrangement is responsible (15). In collagen, lines originate in a large scale periodic variation of density along the fiber axis (21), while in nylon and polythene some maxima are observed for which no convincing explanation has been given (22, 23). In all cases, however, some reasonably regular, large scale, periodic structure is apparently present.

The continuous low angle scattering has a different origin. No periodic arrangement need be present. All that is required is that large scale discontinuities be present in the material, such as discontinuities in the refractive index for x-rays, which generally arise from discontinuities in density. If regions bounded by such discontinuities are present, then a continuous low angle scattering will be observed. The intensity of this scattering will, among other things, depend on the magnitude of the change in refractive index, while the rate of change with angle of scattering will depend on the size and shape of the regions. This sort of scattering has been observed, for example, in rubber (17) and in cellulose fibers (24).

Both types of low angle scattering are affected by orientation in the specimen. In orientated tobacco mosaic virus specimens the low angle scattering occurs at right angles to the direction of orientation, while in collagen the maxima are found in the direction of the fibers. Similarly, in stretched rubber the intensity distribution is different parallel to than perpendicular to the direction of stretching.

Particularly for materials of interest to biochemists, where large scale oriented structures may well be the rule rather than the exception, it would appear that the low angle x-ray scattering studies may often give useful information. The few studies that have thus far been carried out have certainly yielded interesting results.

## PROTEINS

*Crystalline proteins.*—The reviewer has been actively engaged in x-ray studies of proteins and has had ample opportunity to acquire some convictions about this subject. Particularly for this reason, he is resolved not to write a review which will become an essay in numerology (25, 26) or a discussion of the aesthetics of geometrical patterns (27, 28). The data available are scanty, and one should always attempt to extract from them as much as possible. However, the need is really for more experiments and better data rather than for elaborate theories based on inadequate data.

Spiegel-Adolf and collaborators have been engaged in the study of the denaturation of proteins and its possible reversal (29, 30), and in the study of tuberculin proteins (31) and of iodinated amino acids and proteins (32). In all these papers, the least sensitive of all x-ray methods was used, namely, the wide angle scattering from unoriented specimens. The conclusions should therefore be treated with reserve, particularly those of a quantitative nature. Thus it is difficult to see how spacings can be given to four significant figures (32) when a 1 mm. diameter pinhole system, a 37.7 mm. specimen-to-film distance, and unfiltered copper radiation are used. Similarly, their computation of molecular sizes (31) based on line breadths cannot be taken seriously. No corrections are apparently made for the instrumental contribution to the line breadth. Moreover, the wide angle lines considered in this paper are certainly composite in origin and much of the observed breadth could be due to this composite nature. Indeed it is probable that the material studied is not crystalline and hence the reflections cannot be treated from a line breadth computation point of view as Bragg reflections can.

Similar criticisms can be made of the work on protein denaturation (29, 30). It is difficult to see how a diagram containing two or three diffuse halos can be used to characterize the state of a material as complex as a protein. More complex diagrams can be attained under different experimental conditions. Thus crystalline horse serum albumin, wet and dry, has been studied by the writer (23) by both single crystal and powder techniques. The wet diagrams, as is always the case for proteins, are much more detailed than the dry diagrams. The cells in both cases are hexagonal,  $a = 96.7 \text{ \AA}$ ,  $c = 145 \text{ \AA}$  for the wet (in 2.4 *M* ammonium sulfate solution) and  $a = 74.5 \text{ \AA}$ ,  $c = 130 \text{ \AA}$  for the air dried crystals. Powder diagrams of the wet crystals showed some sharp lines at low angles, but even then it was evident



that only a few of the very strong reflections were to be observed in the powder diagram.

In sharp contrast to these powder diagram studies is a series of three short papers by Perutz (33, 34) and Boyes-Watson *et al.* (35). These are a continuation of earlier work by Bernal and co-workers (36). Perutz has undertaken the ambitious task of determining directly as much of the structure of haemoglobin as can be done with x-rays. He used sharply collimated, highly filtered, x-ray beams, comparatively large specimen-to-film distances, single crystals as specimens and, most important of all, a minimum of assumptions in the interpretation of the x-ray diagrams. In the first of these papers (33), crystals of horse methaemoglobin were studied in various states of hydration. Patterson's projections were made on the "b" plane (since this is the plane in which maximum change with water content occurs) and the Patterson plots for the different degrees of hydration were compared (37). Tentatively, the haemoglobin molecule is deduced to be a platelet 36 Å thick, 64 Å long, and somewhat shorter in the third dimension. In the 36 Å direction, the molecule consists of either one or two sheets.

In the second paper (34), oxyhaemoglobin was studied. Vector maps (Patterson diagrams) are made and significant resemblances are noted to the methaemoglobin maps. This is considered reasonable, if one assumes that no substantial difference exists in the general molecular architecture of oxyhaemoglobin and methaemoglobin. Use is made of the known optical characteristics to arrive at some conclusions about the arrangement of the molecules in the crystal.

In the third paper (35), use was made of the change in intensity of reflections with changing state of hydration to determine the signs of the amplitudes of the (001) reflections of methaemoglobin. This choice of signs was further checked by comparing the intensity from two different crystals. In one, the intermolecular liquid was ammonium sulfate solution; in the other, the crystal was salt-free. Once the signs were determined, it was possible to compute a one dimensional Fourier projection, projected onto a line normal to the flat haemoglobin layers. This gave a four peaked contour map which suggested the presence of only one layer of water per protein molecule. It is then concluded that the water layer is essentially intermolecular and that the haemoglobin molecule consists of four equal layers of scattering matter.

These three papers are significant not because of the results—

for while the analysis is logical the conclusions are not necessarily definitely correct and should merely be considered as reasonable possibilities—but because they represent an attempt to collect the best experimental x-ray data obtainable from these materials followed by an analysis, reasonably free from preconceptions, aimed at letting the data tell what they can rather than trying to force the data to prove some theory which they are inadequate to do.

Ferritin and apoferritin have been studied using the powder method (38). Both are cubic and crystallize in face-centered cells of identical size. The relative intensities of the lines are different in the two cases. It is concluded that the protein, which is the same in both cases, determines the packing.

Ribonuclease has been found to crystallize in two different forms (23, 39, 40) one orthorhombic, the other monoclinic. The orthorhombic cell contains four molecules, the monoclinic two. This protein may prove to be a good one to study more intensively because of its comparatively small size, its occurrence in two different crystal forms, and the fact that the dry crystals give the best dry x-ray diagrams obtained thus far.

$\gamma$ -Chymotrypsin has also had preliminary studies made of it (23, 40). It is tetragonal with eight molecules per unit cell.

*Fibrous proteins.*—The review in 1939 (41) and 1942 (42) devoted considerable space to the fibrous proteins. Huggins has recently reviewed this field very comprehensively (28). He does not appear to like the structures, both new and old, suggested by Astbury for the fibrous proteins, and he proposes alternative structures of his own.

The controversy over the structure of the fibrous proteins has followed the pattern set in similar controversies concerning all other fibrous biological materials in which attempts have been made to give detailed structures. In all cases, the data were inadequate to determine a structure, but, nevertheless, structures were proposed. Theories are a good thing when they lead to better experiments and improved data; they are sterile when they merely result in more theories. The reviewer feels strongly that, particularly in the application of x-ray methods to the study of biological materials, too much emphasis has been placed on over-extended interpretations of the data. Because biological materials are often refractory, the tendency has been to accept the data which are easily obtainable and to try to make the most of them. This course has led to much controversy

and not to much useful knowledge. It would be much better to go only as far in the interpretation as the data warrant—even if that is not very far—and to refine the apparatus and technique to obtain more significant data.

Fibrinogen and fibrin can be prepared in oriented preparations (43). They belong to the keratin-myosin group of fibrous proteins. Both give the  $\alpha$  pattern, but some  $\beta$  pattern can be obtained in fibrinogen by laterally squeezing the oriented preparation, and in fibrin by stretching.

Keratin has been the subject of several papers. The interest has been on the long spacings (low angle scattering). By using better defined x-ray beams, longer specimen-to-film distances, and carefully selected oriented specimens, much more comprehensive data were obtained. The fiber diagram in addition to the usual gross features exhibited a fine structure particularly along the meridian (fiber axis) (26). Astbury's side chain spacing is also composite (12). Along the meridian, spacings as long as 66 Å appear. These are interpreted as orders of a period along the fiber axes of either 658 Å or 198 Å. A rather involved discussion is given of the data in terms of Bergmann & Niemann's (44) and of Astbury's (25) ideas of the stoichiometry of the proteins.

A second paper (45) in which only the meridian spacings are measured for porcupine quill tip and for feather rachis indicates fundamental periods of 198 Å and 95 Å for the porcupine quill and the feather respectively.

Collagen has been studied extensively using low angle scattering techniques (16, 21, 46, 47). Beef tendon gives about thirty orders of a fundamental spacing of 640 Å (16). Electron microscope photos of beef tendon show a striated periodic structure along the fibrils (46). The period varies from fibril to fibril, periods from 500 Å to 900 Å having been observed. This agrees well enough with the x-ray observations inasmuch as the extreme treatment given the specimens in preparing them for the electron microscope would be expected to affect the absolute value of the periods observed.

Clam muscle fibrils in the electron microscope show a similar striated structure with periods between 290 Å and 470 Å with an average value of 360 Å (47). X-ray low angle measurements indicate a period of 700 Å. This difference is considered real by the authors.

In an extensive paper (21), Bear gives a detailed account of his low angle studies of collagen. The large period along the fiber axis

together with the principal wide angle features are given for twenty-six different specimens. Where observed (twenty-three cases), the large fiber period was of order of 600 Å. The physical condition of the specimen affects the period, wet specimens having larger periods than dry or "tanned" specimens. It is also suggested that just as in the case of tobacco mosaic virus (15), the low angle scattering does not necessarily change with changing conditions in the same way as does the wide angle scattering. No good correlation between the wide angle and low angle scattering could be offered.

*Protein fibers.*—Palmer & Galvin (48) have produced a denaturation of a globular protein, egg albumin, by treatment with a detergent. Highly oriented, very strong fibers were made from the denatured protein which give the  $\beta$  pattern. Senti *et al.* (49) have produced similar fibers, also giving the  $\beta$  type x-ray diagram, from casein,  $\beta$  lactoglobulin, haemoglobin, ovalbumin, edestin, zein, and peanut and soybean proteins. In this work the denaturation was accomplished by heating a water-protein mixture. These results suggest strongly that the picture of globular proteins as being a folded configuration of a polypeptide chain is substantially correct.

#### CELLULOSE

A group of letters in *Nature* (50, 51, 52) is good evidence that the structure of cellulose is still not entirely settled. A proposed slight structural change by Pierce (50) is criticized by Astbury and by Cox (51, 52).

#### STARCH

The work in this field has made substantial progress in the last three years. Bear & French (53) using the powder technique obtained good diagrams of various starches. They deduced unit cells for the A and B type starches, but later work (54) has shown that they were only partly successful in this attempt to base a unit cell on x-ray data from an unoriented specimen of a complex material.

Rundle and co-workers in two papers (55, 56) report on the optical properties of starch and starch-iodine complexes. In the first of these (55), solutions both of the branched and unbranched fractions of starch were examined for dichroism of flow. In the second paper (56), the birefringence and dichroic properties of amylose crystals and of rosettes of amylose were studied, both unstained and stained with iodine. It is concluded that the evidence is in favor of a helical starch chain, and a hexagonal packing of such helices is proposed for the

crystals. Bear (57) obtained diagrams from starch-iodine complexes. He suggested that the data fit a hexagonal packing of helices. Rundle *et al.* (58) also studied amylose-iodine complexes and also suggest a hexagonal unit cell.

Rundle & Edwards (59) studied wet and dry powders of butanol precipitated amylose. The two diagrams show some similarities. Both are considered as possessing orthorhombic unit cells containing two helices and are also pseudo-hexagonal, the hexagonal cells containing four helices. A structure is proposed in which half of the helices run in one direction, the other half in the opposite direction.

In a recent paper, Rundle and co-workers (54) report studies on oriented specimens, both films and fibers of the B modification of starch. Unequivocally, they determine a repeat period of about 10.6 Å along the fiber axis. All of the films can be indexed on the basis of an orthorhombic unit cell for which  $a = 16.0$  Å,  $b = 10.6$  Å,  $c = 9.2$  Å. The  $a$  and  $c$  values agree well with two of the cell constants suggested by Bear & French (53), but there seems to be no connection between the  $b$  value of Rundle, which is the only certain value, and any of the cell constants of Bear.

Amylose fibers with glycerol plasticizer (60) show a period of 7.5 Å. While this is close to the 8 Å period deduced for the helical form (59), this fiber is considered to possess linear chains rather than helices because of the character of the birefringence.

One can see that the studies on starch have followed a fairly conservative and logical course. The earlier workers who obtained not too good diagrams from unoriented specimens were content, and properly so, to use the x-ray data to classify the starches. When better patterns were obtained from unoriented specimens, a not too successful attempt was made to choose a unit cell. Then followed careful optic studies which were most valuable as soon as oriented specimens were examined with x-rays. Not until single crystals or films possessing secondary orientation are studied, however, will it be possible to be sure even of the unit cells of any of the starch fractions or complexes.

#### STEROLS AND RELATED COMPOUNDS

The outstanding x-ray study in recent years of materials of biochemical interest is Crowfoot's report on steroid structures (6). While partly a review, this paper also presents much heretofore unpublished work. Following Bernal (4, 5), use was made both of single crystal x-ray and optic data. Cholesteryl iodide crystallizes in two

different forms and by the use of Fourier methods, the complete structure of one of these forms was determined. As a result, much could be said about this sterol molecule. To quote:

The side chain continues the general line of the sterol ring system and is shown to be attached in the *cis* position to the methyl group at C13, a point about which there has been some argument. The stereochemical relation of the atoms C13, C16, C17, C20, C21, and C22 are now well established.

Other stereochemical questions were also answered by this work. The choleic acids and heart poisons were also discussed in detail. This paper is well worth reading not only for the results—which are impressive—but because of the way the experimental data have been evaluated. The data are good—one now knows no way in which better data could be obtained from these materials. In the interpretation, despite the obvious desire of the author to answer many pressing chemical questions, no attempt was made to “squeeze” more out of the data than was warranted. This work provides a striking contrast, unfortunately, to many other x-ray papers on biochemical materials.

#### BONE

Considerable light on the identification of the structure of the inorganic bone salt has been cast by x-ray diffraction data. The chemical composition of bone is known to vary slightly under normal conditions, and somewhat more under abnormal conditions. Thus the crystalline material may be such as to be able to retain its crystalline form with slight changes in its chemical composition.

In 1926, De Jong (61) made several Debye-Scherrer diffraction patterns on powdered bone. He compared the patterns thus obtained with that of fluorapatite, and found them to be very similar. This fact indicates that the inorganic matter of bone has an apatite-like structure. The apatite minerals may vary somewhat in composition and yet give similar diffraction patterns. Taylor & Sheard (62), in 1929, ran a similar study and showed that normal and pathological bone and several apatite minerals gave similar diffraction patterns, whereas brushite,  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  gave a pattern quite distinct from the others. In 1931, Roseberry, Hastings & Morse (63) presented further evidence on similar lines. They indicated that calcium carbonate and calcium acid phosphate, as such, are not present, and that bone resembles dahllite (carbonate apatite) more than the other apatites they studied, i.e., fluorapatite, chlorapatite, dahllite, and tertiary calcium phosphate.

Clark (64) presented x-ray diffraction patterns on specimens of bone sections, whereas previous workers had confined their work to

bone powder. The lines she obtained were in agreement with those of Roseberry, Hastings & Morse (63). Certain of the lines she obtained were arched, which indicates orientation of the apatite-like crystals along the longitudinal axis of the bone. Shauman (65) ran diffraction patterns on ignited bone and reported an apatite structure with the inorganic substance in high dispersion. On unignited bone, the diffraction patterns of rachitic bones showed the lines due to organic matter with much greater intensity than normal bone. Clark, Bucher & Lorenz (66) also ran x-ray diffraction pattern studies on bone, cartilage, and other tissues.

In 1931, Hendricks, Hill, Jacobs & Henderson (67) made a careful and critical study to determine the exact nature of the inorganic substance of bone. On the basis of chemical experiments and x-ray diffraction data, they came to the conclusion that bone is essentially a carbonate apatite or a carbonate hydroxyapatite,  $\text{Ca}_{10}\text{CO}_3(\text{PO}_4)_6 \cdot \text{H}_2\text{O}$ . Steaming and ignition tends to change the structure somewhat. However, an important point of this paper may be criticized, namely, the assumption that calcium oxide formed from amorphous calcium carbonate by ignition should be crystalline. In addition, would calcium carbonate, if adsorbed on the surface of the crystals, yield crystalline calcium oxide on ignition? In 1932, Hendricks, Jefferson & Mosley (68) studied several of the apatite minerals and showed that fluorine in fluorapatite can be isomorphously replaced by carbonate, sulfate, silicate, or hydroxyl groups, and by oxygen, chlorine, bromine, and iodine ions. The inorganic portion of bone proximates the formula  $\text{Ca}_{10}\text{CO}_3(\text{PO}_4)_6 \cdot x\text{H}_2\text{O}$  and forms a solid solution with hydroxyapatite. The powder patterns of the apatite are very similar, and the lattice dimensions do not differ very much; however, it is possible to distinguish between them.

Klement & Trömel (69) and Bredig (70) presented strong evidence that the structural inorganic substance of bone and teeth was hydroxyapatite rather than carbonate apatite. Bredig compared powder patterns of several apatites, including staffelite and synthetic carbonate apatites and hydroxyapatite, and stated conclusively that the inorganic substance of bone is hydroxyapatite. A few years later, Klement (71) claimed to have synthesized the inorganic portion of bone by precipitating hydroxyapatite from solution in the presence of carbonate. Gassman (72) disputed Klement's work and stated that it was not in agreement with analytical results. He cited an experiment to show that no free calcium carbonate exists in bone. However, this is still a doubtful point.

In an x-ray diffraction study on teeth, Bale, Le Fevre & Hodge (73) identified the main substance as hydroxyapatite with occluded or adsorbed carbonates. A few years later, Bale (74) showed that the unit cells of the inorganic substance of bone, tooth enamel, dentine, hydroxyapatite, and tricalcium phosphate hydrate are identical within the range of experimental error. The inorganic substance of bone and tooth enamel tends to approximate the structure of hydroxyapatite. He suggested that the carbonate present in these substances does not form an integral part of the lattice, but is mainly present in boundary regions.

More recently, Hendricks & Hill (75) wrote a provoking article throwing the subject of the constitution of the inorganic substance of bone into dispute again. Their interpretation of the evidence is that bone contains a hydrated tricalcium phosphate type of compound instead of hydroxyapatite, as is widely accepted, and that sodium and carbonate are essential constituents of this compound. They suggest the average composition of the unit structure of bone to be  $\text{Ca}_{8.50}\text{Mg}_{0.25}\text{Na}_{0.19}(\text{PO}_4)_{5.07}(\text{CO}_3)_{1.24} \cdot 2\text{H}_2\text{O}$ .

In 1934, Clark & Mrgudich (76) reported a study on the effect of rickets on the structure of bone. Their work is in agreement with previous work (63, 64). They found the preferred orientation of certain lines, as reported by Clark (64), and discovered that in rickets this orientation disappears and the diameter of the innermost ring, indicative of amorphous material, increases. This fact indicated that the organic material of bone had broken down as a result of the rickets. Saupe (77) presented results of the effect of age, rickets, osteomyelitis, and repair of broken bone, on bone structure.

Struler (78), working with longitudinal sections of hollow bones, found the crystallites arranged in regular fashion, fiber diagrams being obtained. The organic matter had been removed by ignition. The arrangement of the crystals was one of a multiple spiral fiber.

Henschen (79) gives a detailed account of the composition and structure of bone, and the changes with age and fatigue as revealed by x-rays. Korsi (80) ran an x-ray study of the organic and inorganic portion of bone. His findings on the inorganic portion are not new. He states that collagen in cartilage is present as micro crystals whose arrangement depends on the type of cartilage. Lamarque (81), in an x-ray diffraction study of human bone, states that the collagen determines the orientation of the inorganic substance, and that at each point of the bony tissue, the ternary axis of the apatite-like compound seems to be parallel to the local direction of the polypeptide



chain of the collagen. Reed & Reed (82), in a study of the effect of rickets and of healed rickets on the structure of bone, discovered that the disorientation of the inorganic portion of the bone which occurs during rickets cannot be reversed on healing the rickets. These authors also show that bone and apatite give very similar, although not identical, diffraction patterns. Dallemagne (83, 84) reported, on the basis of an x-ray diffraction investigation, that the inorganic material of bones consists of a mixture of  $\alpha$ -tricalcium phosphate and calcium carbonate, and that on ignition ( $900^\circ$ ) an apatite is formed.

It is apparent that there is still considerable confusion regarding the exact nature of the inorganic portion of bone, and more exacting work on the structure of the various apatites and calcium phosphate compounds may help out in this respect. At present, it is very difficult to get a sharp x-ray diagram of bone without treatment which may introduce an alteration of structure, and this is probably the principal cause of confusion, considering the fact that most of the possible structures give similar diffraction patterns.

There seem many ways in which x-ray work can assist in this field in the future. It would appear that more work should be done on intact bone. A micro x-ray technique (17), combined with microradiography (85) applied to bone sections, should be useful. Low angle x-ray work (23) indicates that bone gives an oriented low angle x-ray scattering. This at present appears to be of the continuous type. A study of this scattering should give some information about the large scale structure (50 Å to 500 Å) present in bone.

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DEPARTMENT OF CHEMISTRY  
POLYTECHNIC INSTITUTE OF BROOKLYN  
BROOKLYN, NEW YORK

# THE CHEMISTRY OF THE STEROIDS

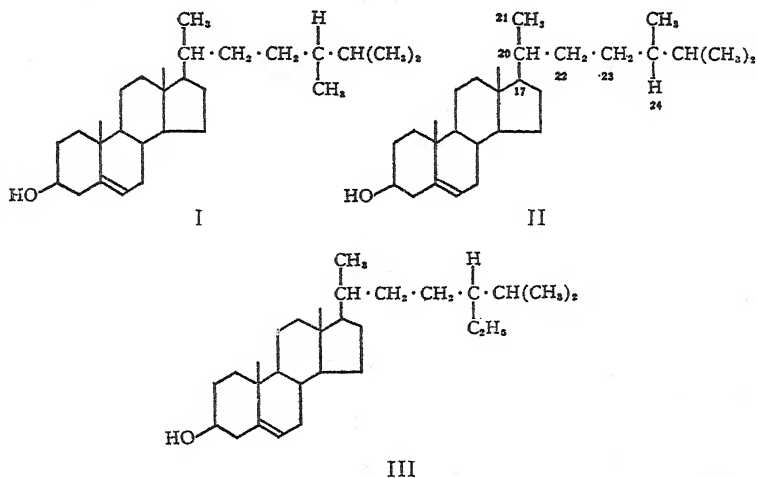
BY WILLIAM L. RUGH

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This review covers primarily the publications available in the past year on the chemistry of the natural sterols, bile acids, and steroid hormones. The rapid advances in the separation of the urinary steroids and the clinical interpretation of these results are reviewed in the section on hormones. The sapogenins are also treated separately.

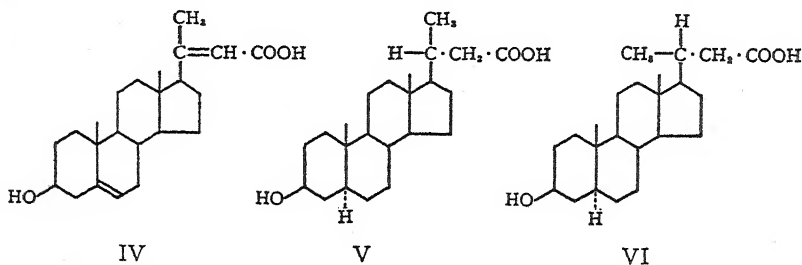
## ISOLATION AND STRUCTURE OF NATURAL STEROLS

The isolation of a pure homogeneous sterol from the usual sterol mixtures obtained from natural sources is often a problem of more than usual difficulty. The sterols differ in the number of carbon atoms in the side chain and in the number and position of double bonds in the nucleus and the side chain. A new type of isomerism in natural sterols due solely to a difference in the optical configuration on C-24 was first found in the case of campesterol (I) and 22,23-dihydrobrassicasterol (II) (1). The latter sterol has not yet been isolated from natural sources but was prepared from brassicasterol by reduction of the side-chain double bond (2).



The second example of the C-24 epimeric type of isomerism has now been shown to occur in the case of  $\beta$ - and  $\gamma$ -sitosterol, both of

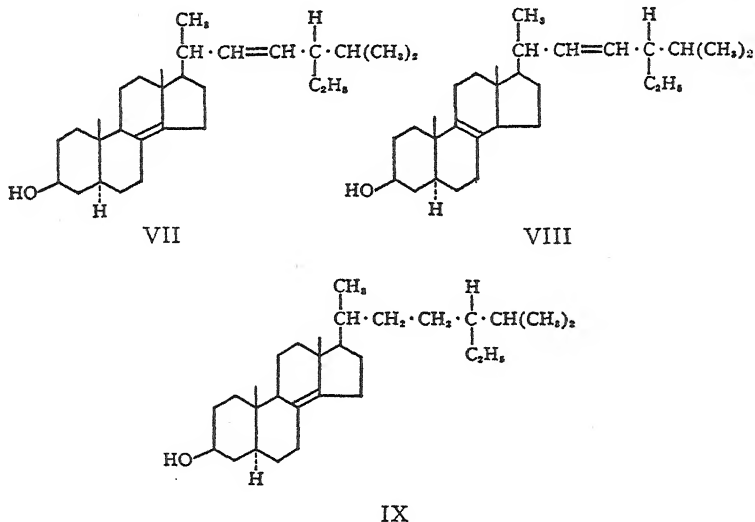
which occur in the sterols from soybeans (3). The acetate of  $\beta$ -sitosterol (III) on oxidation with chromic acid in acetic acid yielded acetone and a dextrorotatory ketone characterized as the 2,4-dinitrophenylhydrazone and the semicarbazone. The latter ketone was identified as 6-methyl-5-ethyl-heptanone-2 by comparison with the synthetic *dl*-ketone (4). The determination of the optical homogeneity of the natural ketone awaits the resolution of the synthetic ketone into its two antipodes. In like manner,  $\gamma$ -sitosterol on oxidation yielded acetone, isolated as the 2,4-dinitrophenylhydrazone, and an oily laevorotatory ketone characterized as the semicarbazone, m.p. 140–42°. Due to the small amount of material and the temporary discontinuance of the work, careful comparison of the x-ray data and mixed melting point diagrams with the synthetic *dl*-ketone was not possible as in the case of the dextrorotatory ketone from  $\beta$ -sitosterol. However, the probability remains that  $\gamma$ - and  $\beta$ -sitosterols are isomers differing only in the configuration of the ethyl group on C-24. Because oxidation of the side chain to the ketone destroys another center of asymmetry at C-20, there remains still the possibility of this same type of isomerism at C-20 and C-17 being a contributing factor in the isomerism of this pair of sterols. For campesterol (1), this possibility was excluded by the oxidation of campestanol acetate and the isolation of 3( $\beta$ )-hydroxynorallocholanic acid (V) and the derived 3( $\beta$ )-acetoxymethyl ester, which were identical with the corresponding compounds obtained from  $\beta$ -cholestanol acetate. This evidence was greatly strengthened by the recent preparation of 3( $\beta$ )-hydroxy-20-isonorallocholanic acid



(VI) and the normal epimer (V) by the reduction of  $\Delta^{5:6,20:22}$ -3( $\beta$ )-hydroxy-norcholadienic acid (IV) (5). The normal acid differs structurally from the 20-iso acid only in the optical configuration of the groups on C-20. The physical properties of the corresponding acids, methyl esters and 3-acetoxy-methyl esters differ markedly, particularly

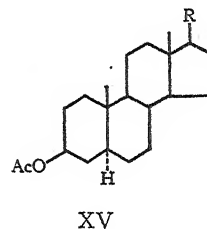
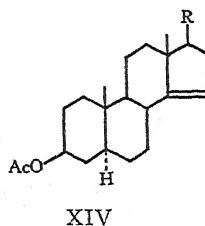
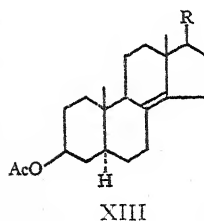
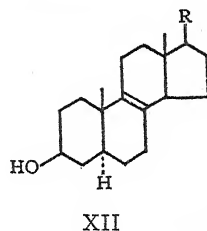
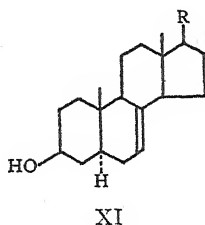
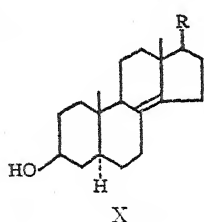
in their melting points. Thus confusion is not possible in comparing these two acids or their derivatives with the corresponding compounds obtained from the oxidation of a sterol of undetermined structure. The evidence for the existence of two series of saturated derivatives in the group of the digitalis, scilla, and toad poison genins differing in configuration on C-20 has been reviewed (5). However, no positive evidence for isomerism due to the optical configuration of the groups on C-17 or C-20 in the natural sterols is known to the writer. The third example of C-24 isomerism and the first case of its occurrence in an animal sterol will be discussed in connection with the structure of the starfish sterols which follows.

Prior to 1940 all naturally occurring unsaturated sterols of known structure, with the exception of  $\alpha$ -dihydroergosterol, possessed a double bond in position 5:6 of ring B. In that year the unsaturated sterols, zymosterol from yeast (6) and  $\alpha$ -spinasterol from higher plants (7), were shown not to have a 5:6-double bond. The structure (VII) assigned to spinasterol (7) has been modified on the basis of a study of its oxidation products (8) to formula (VIII) in which the double bond is placed between C-8 and C-9 instead of between C-8 and C-14 of ring C. The reported isolation (9) of three isomeric  $\alpha$ -,  $\beta$ -,



and  $\delta$ -spinasterols, all of which are reduced to  $\alpha$ -stigmastenol (IX), might be explained on the basis of isomerism of the nuclear double

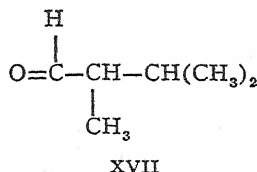
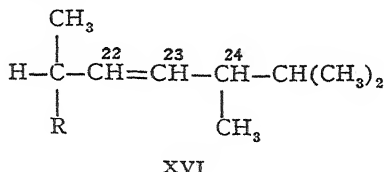
bond although there is no experimental evidence on this point. The recent investigation (10) of the unsaponifiable fraction from the starfish, *Asterias forbesi*, showed that the sterol is a mixture which resisted all attempts to resolve it into pure compounds either by repeated recrystallization or by chromatography. Catalytic reduction in the presence of platinum of the acetates of any of the many fractions obtained gave the same product,  $\alpha$ -stellastenol acetate having a constant melting point and rotation. Thus the cyclic double bond is attached to C-8 as an  $\alpha$ -,  $\gamma$ -, or  $\delta$ -double bond. The starfish sterols are the first examples of an unsaturated animal sterol not having a double bond in the 5:6-position.



The presumption is that the mixture of starfish sterols may contain the  $\alpha$ -,  $\gamma$ -, and  $\delta$ -stenols (X, XI, XII) with, in some cases, an additional double bond in the side chain R. Hydrogenation with platinum in acetic acid, in addition to saturating the side chain, isomerizes the  $\gamma$ - and  $\delta$ -stenyl acetates to  $\alpha$ -stellastenyl acetate (XIII), which contains the resistant double bond. Isomerization of the  $\alpha$ -stellastenyl acetate with hydrochloric acid gave  $\beta$ -stellastenyl acetate (XIV), which was then reduced to stellastenyl acetate (XV).

The starfish sterols are also unique in that they are the first  $C_{28}$  sterols to be found in animals, with the exception of ergosterol which occurs in snails and earthworms and which may well be of exogenous origin. The  $C_{28}$  carbon skeleton was determined by analysis of the

*m*-dinitrobenzoate of stellastanol and the fact that ozonolysis of the original sterol mixture followed by treatment with 2,4-dinitrophenylhydrazine gave the 2,4-dinitrophenylhydrazone of methylisopropylacetaldehyde (XVII). The optical rotation of the hydrazone was  $+14.1^\circ$ , whereas the rotation of the corresponding hydrazone of methylisopropylacetaldehyde obtained from ergosterol was  $-37.7^\circ$ . Hence the side chain of the di-unsaturated stellasterol has the structure XVI.



Stellasterol, therefore, has an optical configuration on C-24 similar to campesterol and opposite to that of ergosterol and this is the first time this C-24 type of isomerism has been found in sterols from animals.

Stellastanol obtained from the acetate (XV) differs in its physical constants from the known pair of  $\text{C}_{28}$  stanols epimeric with regard to the configuration C-24, ergostanol and campestanol (Table I).

TABLE I (10)

COMPARISON OF SATURATED STEROLS OF THE FORMULA  $\text{C}_{28}\text{H}_{50}\text{O}$ 

Name	Sterol		Acetate		<i>m</i> -Dinitrobenzoate	
	m.p.°C.	$\alpha_D$	m.p.°C.	$\alpha_D$	m.p.°C.	$\alpha_D$
Stellastanol ....	143	+22	139	+13	204	....
Ergostanol ....	144	+15	145	+ 6	203	+13
Campestanol ...	147	+31	147	+18	198	+22

There seems to be a distinct possibility that stellastanol is not a new isomer of ergostanol or campestanol, but a constant and difficultly separable mixture of the two. The only other alternative based on the available evidence would require the assumption of epimerism at one or more additional asymmetric centers either in the nucleus or at C-17 or C-20. The possibility that stellastanol is a mixture of C-24 epimers is supported by the lower melting points of the stanol and its acetate and the fact that their optical rotations are intermediate in value to those of the known epimeric pair. Furthermore, the low posi-

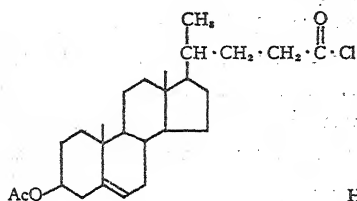


tive value of the observed optical rotation of the 2,4-dinitrophenylhydrazone of the methylisopropylacetaldehyde compared to its negative antipode obtained from ergosterol may be the true value rather than that due to a partial racemization of the aldehyde during its isolation. From similar evidence, the presence of a mixture of C-24 epimers in brassicasterol was suspected (11). The isomeric 24-isobrassicasterol (22:23-dehydrocampesterol) has not yet been isolated.

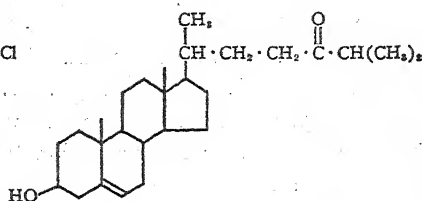
With regard to the nomenclature of C-24 isomers, the term 20-isonorallocholic acid has been used to denote a derivative of cholesterol differing from the normal configuration on C-20 (5). An extension of this type of nomenclature might be applied to C-24 isomers whereby campestanol would be called 24-isoergostanol or ergostanol called 24-isocampestanol. Since carbon atom 24 in the side chain of cholesterol is not asymmetric, it is difficult to find a common term to denote the two series of natural sterols differing in their optical configuration on C-24. Perhaps the two series could be differentiated by reference to the optical rotation of the ketone derived from the fission of the side chain between C-17 and C-20. In this manner any confusion due to the possibility of epimerism of the original sterol on C-17 and C-20 is removed by the elimination of the C-20 center of asymmetry. Thus campestanol could be called 24-*dextro*-methylcholestanol and ergostanol 24-*laevo*-methylcholestanol; likewise,  $\beta$ -sitostanol becomes 24-*dextro*-ethylcholestanol and  $\gamma$ -sitostanol 24-*laevo*-ethylcholestanol. In the preceding structural formulae the arbitrary convention has been adopted of writing the C-24 alkyl group below the side chain in the 24-*dextro* series [campesterol (I),  $\beta$ -sitosterol (III), stellassterol (XVI)] and above the side chain in the 24-*laevo* series [22,23-dihydrobrassicasterol (II),  $\gamma$ -sitosterol, ergostanol]. Further work is, however, necessary on the optical rotations of the ketones and their derivatives obtained from the chromic acid cleavage of the entire side chain and the aldehydes and their derivatives obtained from fission of the unsaturated side chains by ozonolysis of the double bond between C-22 and C-23. The isolation of the 3( $\beta$ )-hydroxynorallocholic acid or its epimers would also be necessary in each case to fix the steric relationship of groups attached at C-17 and C-20.

A promising method of attack on the problem of C-24 isomerism of the side chain of sterols has been opened by the synthesis of 24-ketocholesterol (XIX) from the reaction of 3-acetoxy- $\Delta^5$ -cholesterol chloride (XVIII) with diisopropyl cadmium and the reduction of the ketone to 24-hydroxycholesterol (XX) with aluminum isopropylate

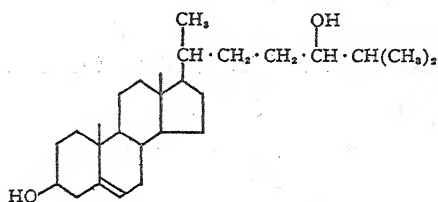
(12). The conversion of these compounds into naturally occurring sterols has not as yet been reported.



XVIII



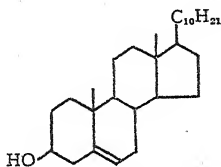
XIX



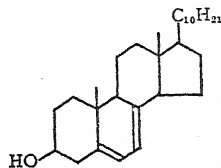
XX

A new approach to the investigation of the homogeneity of sterols and their separation in complex mixtures has been studied in the case of tall-oil sitosterol and the wheat-germ sterols. The method consists in oxidizing the sterol mixture to the 3-ketones with the Oppenauer reagent and the separation of the ketones by chromatography on alumina. The method gives far better separations than have hitherto been obtained either by fractional crystallization or chromatography of the sterols or their esters (13).

The sterol of the marine sponge, *Sphaciospongia vesparia*, contains in addition to clionasterol (XXI) a small amount of a 7-dehydrosterol. With a view towards identifying this sterol, 7-dehydroclionasterol (XXII) has been synthesized from clionasterol by the usual procedures (14).



XXI



XXII

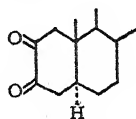
"Cafesterol" from coffee beans has been shown definitely not to have a steroid structure, and it has been suggested that the name of this substance be changed to "cafestol" (15).

The isolation of 22,23-dihydroergosterol from ergot has been claimed. This would be the first time that provitamin D<sub>4</sub> has been found in nature (16). Ambergris has been found to contain coprosterol, epicoprosterol, and some cholesterol (17). The "guanosterol" of Peruvian guano has been shown to be cholesterol. Evidently marine birds do not excrete coprosterol (18). Stigmasterol,  $\beta$ -sitosterol, and a trace of a provitamin D have been isolated from the common bean, *Phaseolus vulgaris* (19).

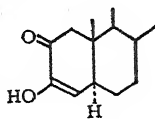
## CHEMICAL TRANSFORMATIONS

### STEROLS

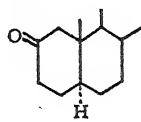
The hitherto unknown 2-ketocholestane and the two epimeric 2-hydroxycholestanes have been prepared starting with  $\Delta^3$ -2-ketocholestene-3-ol (XXIV), the enol form of 2,3-diketocholestane (XXIII) (20). This diketone previously prepared by the selenium dioxide oxidation of cholestane-3-one (21) was also made in the enol form by an application of Kröhnke's method whereby the pyridinium derivative of 2-bromocholestane-3-one was converted to the nitrone



XXIII



XXIV

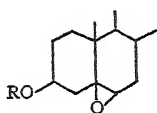


XXV

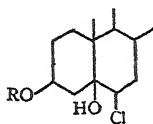
with *p*-nitrosodimethylaniline. The nitrone after treatment with hydrochloric acid gave  $\Delta^3$ -2-ketocholestene-3-ol (XXIV). Hydrogenation of the tosylate of XXIV with Raney nickel gave 2-ketocholestane (XXV). One epimer ( $\beta$  ?) was obtained by hydrogenation of 2-ketocholestane in acetic acid with platinum, and the other epimer 2( $\alpha$  ?)-hydroxycholestane was obtained by reduction with sodium and alcohol. The  $\beta$ (?) epimer gave a precipitate with digitonin. 2-Acetoxycholestane-3-one and the corresponding  $\alpha$ -hydroxyketone are extraordinarily labile substances which undergo several unexpected rearrangements. On catalytic hydrogenation in the presence of platinum 2-acetoxycholestane-3-one yielded, depending somewhat on the solvent, a num-

ber of different products, including cholestane-1-ol acetate and three acetoxycholestanols of unknown structure. From the mixture obtained on Wolff-Kishner reduction of 2-acetoxycholestane-3-one were isolated cholestane, cholestane-4-ol, cholestane-1-ol, and a product which yielded cholestane-2-one on oxidation. Hydrolysis of 2-acetoxycholestane-3-one with alcoholic potassium hydroxide gave  $\Delta^3$ -cholestene-3,4-diol, while potassium carbonate gave 3-hydroxycholestane-4-one together with 2-hydroxycholestane-3-one. These and other rearrangements described show what care is necessary in assigning structures to the reduction products of these  $\alpha$ -hydroxyketones (22).

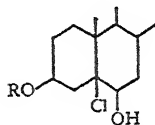
Confusion on the identity of the isomeric  $\alpha$ - and  $\beta$ -cholesteryl oxides XXVI, their esters, and the natures of their transformation products has been clarified by the observation that the previously described " $\beta$ "-cholesteryl oxide and its ester are mixed crystals of the  $\alpha$ -oxide and a new  $\beta$ -oxide or its esters. Fission of the  $\alpha$ -oxide or esters gave derivatives corresponding to formula XXVII and the  $\beta$ -oxide or esters to formula XXVIII (23). The formulas XXVI, XXVII, XXVIII do not represent steric relationships.



XXVI



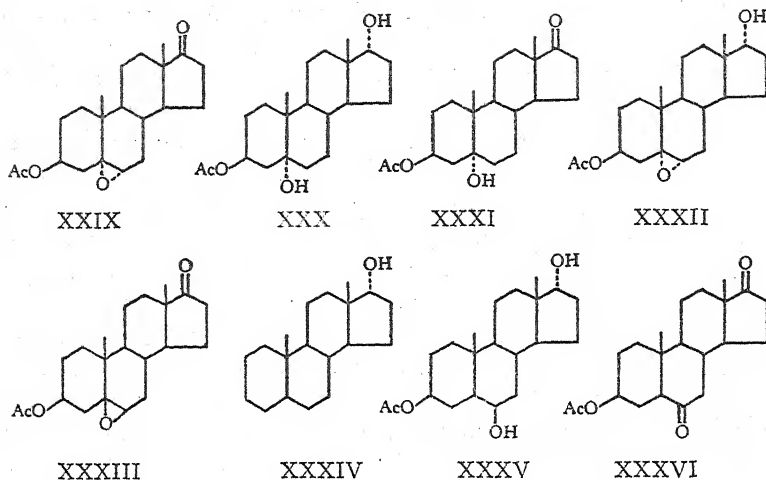
XXVII



XXVIII

In the following paragraph the steric formulas employed by the authors cited will be used. The convention is followed that the  $\alpha$ -oxide ring is written with dotted lines representing the oxide ring as below the plane of the paper. Likewise, the substituents on carbon atoms 5 and 6 are written as in the original (see also references 24 and 25 for alternative conventions).

The reduction of the isomeric oxides of *trans*-dehydroisoandrosterone acetate has been studied (26). The  $\alpha$ -oxide acetate (XXIX) hydrogenated with platinum catalyst in acetic acid gave quantitatively the 3-monoacetate of 3( $\beta$ ),5,17-trihydroxyandrostane (XXX) probably containing a mixture of the 17-epimers which on oxidation with chromic acid gave 3( $\beta$ )-acetoxy-5-hydroxyandrostane-17-one (XXXI). This compound (XXXI) was also obtained directly when the reduction of XXIX was stopped after the absorption of one mole of hydrogen showing that the oxide ring was hydrogenated before the

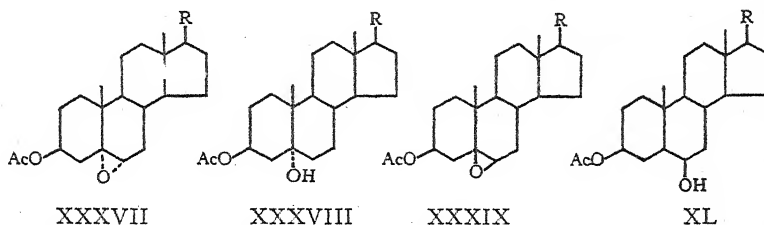


17-keto group. The reverse is true on hydrogenation in alcohol where the product is the  $\alpha$ -oxide of androstene-3( $\beta$ )-acetoxy-17-diol (XXXII). Reduction of the stereoisomeric  $\beta$ -oxide of *trans*-dehydroandrosterone acetate (XXXIII) was very irregular. Hydrogenation with platinum in acetic acid gave androstane-17( $\alpha$ )-ol (XXXIV) with the elimination of the other hydroxy groups. When the reaction was stopped after the addition of two moles of hydrogen, a complex mixture was obtained from which was isolated androstane-17( $\alpha$ )-ol (XXXIV), androstane-17-one, 3( $\beta$ )-acetoxy-6,17-dihydroxyandrostane (XXXV), and 3( $\beta$ )-acetoxy-6,17-diketoandrostane (XXXVI). Hydrogenation of the  $\beta$ -oxide with platinum in alcohol gave androstane-17( $\alpha$ )-ol (XXXIV).

From the standpoint of aglucone synthesis the introduction of the 5-hydroxyl group into the sterol molecule is of great interest. Hence, a study was made of the reduction products of cholesteryl 5:6  $\alpha$ - and  $\beta$ -oxides (27). The separation by fractional crystallization of the pure cholesteryl oxides and their derivatives from the mixed crystals already referred to (23) was accomplished independently by chromatography on alumina. Thus the earlier prediction (28) of the nonhomogeneous nature of the old " $\beta$ " oxide based on x-ray examination has been amply confirmed.

Hydrogenation of the acetate of cholesterol  $\alpha$ -oxide (XXXVII) in acetic acid with platinum catalyst gave 3( $\beta$ )-acetoxy-5-hydroxycholestane (XXXVIII) (formulated as a cholestane and not a copro-

stane derivative) together with some cholestane-3-ol acetate. These compounds together with a cholestanetriol diacetate had earlier been found to be the products of the reduction when a palladium catalyst was used (29). The acetate of the  $\beta$ -oxide of cholesterol (XXXIX) on reduction in acetic acid with platinum follows the irregular type of reduction noted in the androstane series; 3( $\beta$ )-acetoxy-6-hydroxycholestane (XL) and cholestane-3( $\beta$ )-ol acetate were isolated. Also studied was the reduction of cholestane-5:6-oxide and cholestane-4:5-oxide. The former gave cholestane and a mixture of 5- and 6-hydroxycholestanes and the latter a mixture of 4- and 5-hydroxycholestanes.  $\Delta^4$ -Cholestene-3-one has been shown to occur in two forms, one with m.p.  $82^\circ$  and the other (new form) with m.p.  $88^\circ$ , separable by chromatographic methods from the crude Oppenauer oxidation product of cholesterol. The two forms are interconvertible and show a similar behavior to that of progesterone (30).



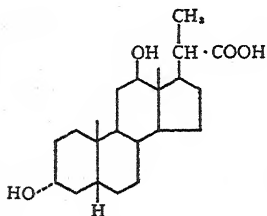
Because of the importance of deuterio cholesterol in metabolism studies the reactions of cholesterol and dihydrocholesterol in dilute acetic acid solutions and in contact with platinum both in the presence and absence of deuterium have been investigated. The products isolated indicate a complex series of hydrogenations and dehydrogenations in addition to the deuterium exchange previously noted (31).

### BILE ACIDS

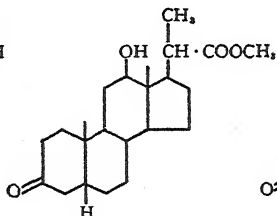
The introduction of oxygen into position 11 of ring C of bile acids and their derivatives is a problem of great importance in the partial synthesis of cortical hormones. The remarkable success in developing a general method to accomplish this has been reviewed earlier (86, 145), and only the most recent advances in this field will be described.

Using bisnordesoxycholic acid (XLI) as the starting material (32), the methyl ester of this acid on acetylation gave the diacetate which on selective hydrolysis with hydrochloric acid in methanol gave

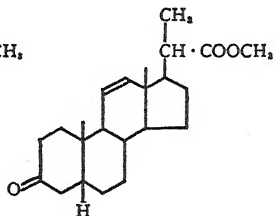
the 12-monoacetate. Oxidation followed by hydrolysis and remethylation gave the methyl ester of 3-keto-12( $\beta$ )-hydroxycholanolic acid (XLII). The benzoate or anthraquinone- $\beta$ -carboxylic acid ester of XLII on thermal decomposition in a vacuum gave the 3-keto- $\Delta^{11}$ -bisanthraquinone- $\beta$ -carboxylic acid methyl ester (XLIII) which on hydrogenation in an alkaline alcoholic solution with Raney nickel followed by acetylation and methylation gave the 3( $\alpha$ )-acetoxy- $\Delta^{11}$ -bisanthraquinone- $\beta$ -carboxylic acid methyl ester (XLIV) together with a smaller amount of the  $\beta$ -epimer. The ester (XLIV) was also prepared starting with bisnordesoxycholic acid, forming the dibenzoate or dianthraquinone- $\beta$ -carboxylate, selectively hydrolyzing the 3-ester group with a special buffer mixture of methanol, phenol, and potassium phenolate, acetylating the 3-hydroxyl and thermally decomposing the 3-acetoxy-12-anthraquinone- $\beta$ -carboxylate. The ester (XLIV) is identical to that earlier prepared by another route (33). The ester (XLIV) treated with bromoacetamide gave the 11-hydroxy-12-bromo ester, which was oxidized with chromic acid to the 11-keto-12-bromo ester. On treatment with zinc dust it gave the methyl ester of 3( $\alpha$ )-acetoxy-11-ketobisanthraquinone- $\beta$ -carboxylic acid (XLV), which on hydrogenation gave the methyl ester of 3( $\alpha$ )-acetoxy-11( $\alpha$ )-hydroxybisanthraquinone- $\beta$ -carboxylic acid (XLVI). By a similar series of reactions followed by hydrogenation, the keto ester (XLIII) gave the same ester (XLV) together with the epimeric 3( $\beta$ )-acetoxy-11-ketobisanthraquinone- $\beta$ -carboxylic acid methyl ester.



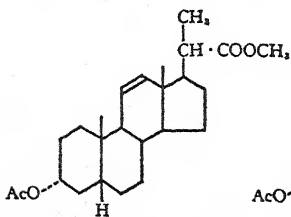
XLI



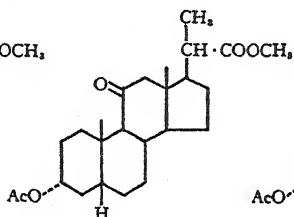
XLII



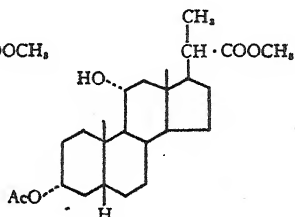
XLIII



XLIV

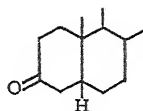


XLV

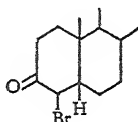


XLVI

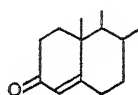
Since the most physiologically active adrenal cortical hormones possess the 3-keto- $\Delta^4$  conjugated structure in ring A (XLIX), a study has been made of the introduction of this system into the desoxycholic acid series (34). Methyl desoxycholate and the corresponding nor-, bisnor-, and etio esters were selectively oxidized to the 3-keto-12-hydroxy acid esters. In certain cases the older method of synthesis (35) involving diacetylation, selective hydrolysis of the 3-acetyl group and chromic acid oxidation followed by hydrolysis of the 12-acetoxy group was used to prepare the 3-keto-12-hydroxy acid esters for comparison. These esters (XLVII) were brominated at position 4 (XLVIII) and then debrominated (XLIX) in pyridine. In this way methyl 3-keto-12-hydroxy- $\Delta^4$ -cholenate and the nor-, bisnor-, and etio homologues were prepared.



XLVII

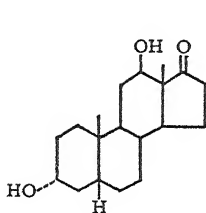


XLVIII

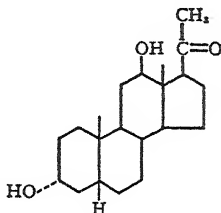


XLIX

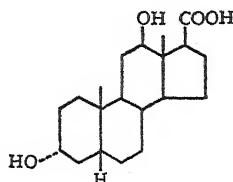
Etiocolane-3( $\alpha$ ),12( $\beta$ )-diol-17-one (L) and its acetate were prepared for use in cortical hormone synthesis by the energetic degradation of the side chain of diacetyl-desoxycholic acid methyl ester by chromic acid oxidation (36). By-products also obtained from this reaction in very low yields were pregnane-3( $\alpha$ ),12( $\beta$ )-diol-20-one (LI) and 3( $\alpha$ ),12( $\beta$ )-dihydroxyetiocolanic acid (etio-desoxycholic acid) (LII). Because of the low yields obtained by direct oxidation of the side chain, further work was done on the preparation of etiocholane-3( $\alpha$ ),12( $\beta$ )-diol-17-one (L) from pregnane-3( $\alpha$ ),12( $\beta$ )-diol-20-one (LI) and etio-desoxycholic acid (LII) (37). These intermediates were available from the stepwise degradation of desoxycholic acid (38).



L



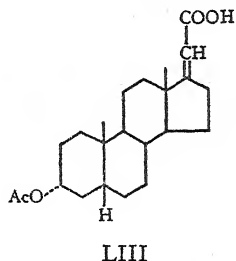
LI



LII

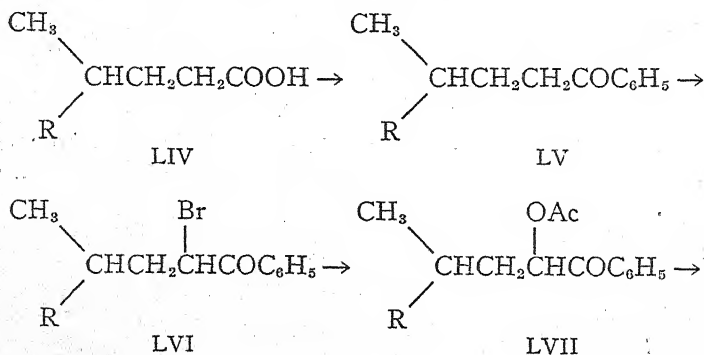


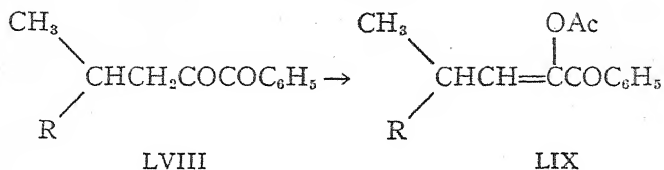
Five known methods and one new method were studied. The best was an adaptation of the bromination route (39) starting with pregnane-3( $\alpha$ ),12( $\beta$ )-diol-20-one (LI), although yields were still unsatisfactory. The diacetate of pregnane-3( $\alpha$ ),12( $\beta$ )-diol-20-one (LI) gave a mixture of the 17-monobromo- and the 17,21-dibromoketones which were then debrominated with alcoholic potassium hydroxide. From the mixture of debromination products was isolated the ethylene derivative (LIII) which on ozonolysis followed by hydrolysis gave the



desired etiocholane-3( $\alpha$ ),12( $\beta$ )-diol-17-one (L) in very low over-all yields.

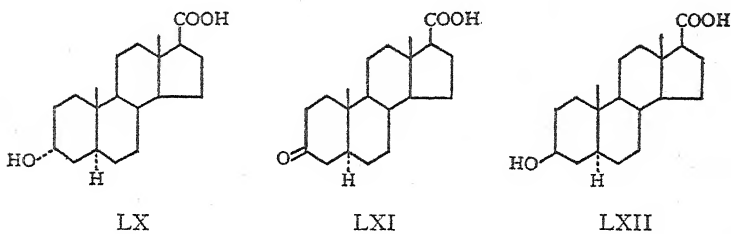
A brief preliminary note on a new procedure for the degradation of the side chain of bile acid derivatives claims higher yields than heretofore obtainable (40). The bile acid (LIV) was converted to the acid chloride which on treatment with diphenyl cadmium gave the phenyl ketone (LV) in 70 to 75 per cent yield. Bromination (LVI) followed by acetolysis gave the 23-acetoxy derivative (LVII) which was hydrolyzed to the  $\alpha$ -ketol and oxidized to the diketone (LVIII) with copper sulfate in aqueous pyridine. The enol acetate (LIX) was then oxidized to the bisnoracid.





This series of reactions applied to the nor bile acids was stated to give very greatly improved yields in the preparation of pregnane-20-one derivatives.

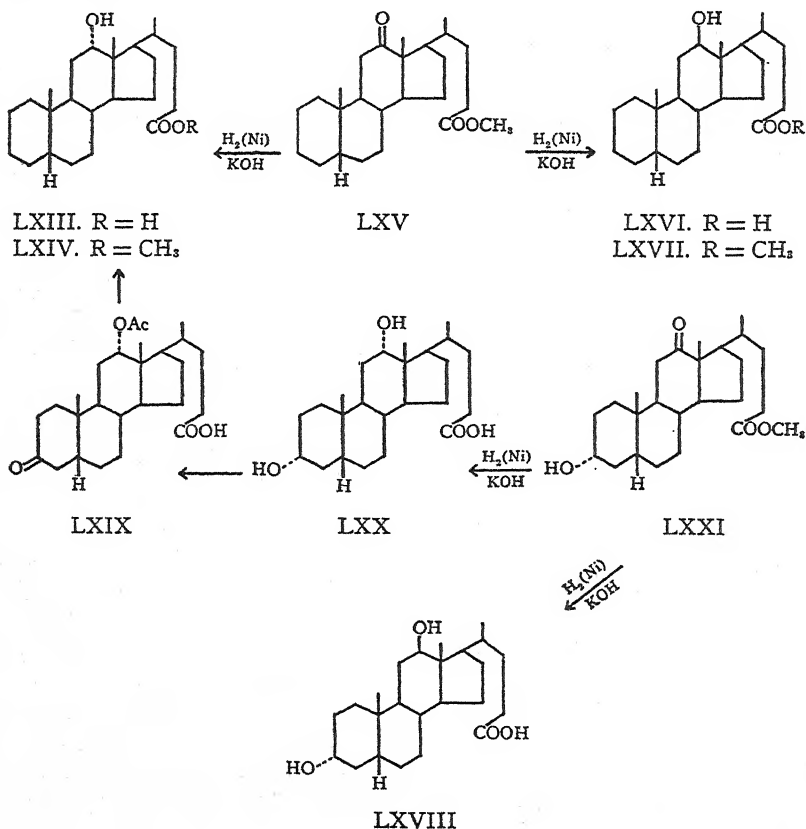
To obtain the previously known 3( $\alpha$ )-hydroxyalloetiocholanolic acid (LX) for synthetic work, a study was made of its preparation from 3( $\beta$ )-hydroxyalloetiocholanolic acid (LXII) (41).



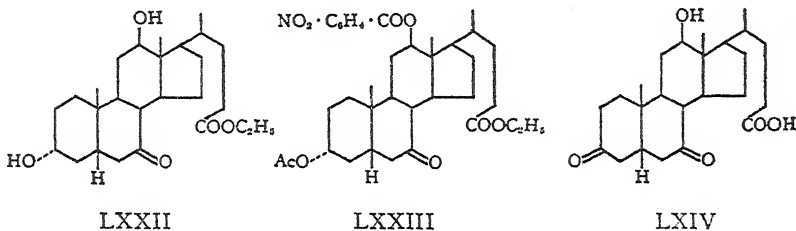
In the first method the epimerization of the 3( $\beta$ )-hydroxyl group was carried out by oxidation of the hydroxy-acid (LXII) to 3-keto-alloetiocholanolic acid (LXI) with chromic acid. The purification of the keto acid was rendered difficult because a tricarboxylic acid was formed as a by-product of the oxidation by the cleavage of ring A. Hydrogenation of the keto acid with platinum catalyst in a mixture of acetic and hydrobromic acids gave in good yield the desired acid (LX) isolated as the methyl ester of 3( $\alpha$ )-acetoxy-alloetiocholanolic acid. The second method of epimerization employed the tosylate of the methyl ester of 3( $\beta$ )-hydroxyalloetiocholanolic acid (LXII) which was refluxed with a solution of anhydrous sodium acetate in acetic acid and gave the methyl ester of the epimeric 3( $\alpha$ )-hydroxyalloetiocholanolic acid (LX) together with the unsaturated methyl alloetiocholenate from which it was separated by chromatography on alumina. Although the over-all yield was not improved, the short tosylate procedure is the method of choice in preparing 3( $\alpha$ )-hydroxyalloetiocholanolic acid.

The hitherto unknown 12( $\alpha$ )-hydroxycholanolic acid (LXIII) has been prepared by two methods (42). In the first, 12-ketocholanolic acid

methyl ester (LXV) (43) was hydrogenated in methanol with Raney nickel, giving a mixture of the known 12( $\beta$ )-hydroxycholanolic acid methyl ester (LXVII) and the new 12( $\alpha$ )-hydroxycholanolic acid methyl ester (LXIV) which gave the free acid LXIII on hydrolysis. The esters were separated by chromatography on alumina. In the second method, 3( $\alpha$ )-hydroxy-12-ketocholanolic acid methyl ester (LXXI) was hydrogenated with Raney nickel in methanol and the mixture of 12( $\alpha$ ) and 12( $\beta$ ) esters formed was hydrolyzed to the free acids (LXX and LXVIII). The acids were easily separated by crystallization, giving first the pure 3( $\alpha$ ),12( $\alpha$ )-dihydroxycholanolic acid (LXX) and then the 3( $\alpha$ ),12( $\beta$ )-dihydroxycholanolic acid (LXVIII). The  $\alpha$ -epimer was converted to 3-keto-12( $\alpha$ )-acetoxycholanolic acid (LXIX) (44), which was reduced by the Wolff-Kishner reaction to the desired 12( $\alpha$ )-hydroxycholanolic acid (LXIII).



Of six possible hydroxy-keto acids having the steric configuration of cholic acid, five were previously known and now the sixth, 12-hydroxy-3,7-diketocholanic acid (LXXIV), has been prepared (45). Cholic acid was oxidized and the product transformed to ethyl 3,12-dihydroxy-7-ketocholanoate (LXXII) which was completely acetylated and selectively hydrolyzed with alcoholic hydrochloric acid to ethyl 12-hydroxy-3-acetoxy-7-ketocholanoate. A *p*-nitrobenzoyl group was introduced in the 12 position, giving ethyl 3-acetoxy-12-*p*-nitrobenzoyloxy-7-ketocholanoate (LXXIII), and a second selective hydrolysis removed the 3-acetyl group without affecting the *p*-nitrobenzoyl radical at position 12. This ester was then oxidized with chromic acid, giving ethyl 12-*p*-nitrobenzoyloxy-3,7-diketocholanoate which on alkaline hydrolysis gave the required 12-hydroxy-3,7-diketocholanic acid (LXXIV). A number of new derivatives of this and other related hydroxy-keto acids were also prepared.

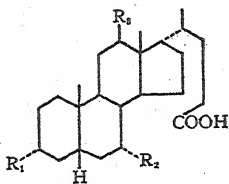


The optical rotations in alcohol and chloroform of a number of bile acids and their derivatives have been correlated with the orientation of the hydroxyl groups using the method developed earlier for sterol derivatives (146, 147). A number of generalizations have been made which are of value in the assignment of various structures to bile acid derivatives (46). The change in molecular rotation of  $[M]_D +175^\circ$  in going from a 7( $\alpha$ ) to a 7( $\beta$ ) bile acid, such as chenodesoxycholic acid to ursodesoxycholic acid, corresponds well with the absolute value of the same change in the cholestane series of  $[M]_D -181^\circ$  but with reversed sign. For example, 3( $\beta$ ),7( $\alpha$ )-dihydroxycholestane has a molecular rotation of  $+214^\circ$ , compared to 3( $\beta$ ),7( $\beta$ )-dihydroxycholestane having a molecular rotation of  $+33^\circ$  (47). The designations  $\alpha$  and  $\beta$  applied to the 7-hydroxy group in the cholestane series were originally assigned arbitrarily.

The relationships in the cholic acid series may be seen in Tables II and III, taken from the original article (46).

TABLE II\*

## CHOLIC ACID SERIES (46)

	$R_1$	$R_2$	$R_3$
Lithocholic acid .....	OH	H	H
Chenodesoxycholic acid .....	OH	OH	H
Desoxycholic acid .....	OH	H	OH
Cholic acid .....	OH	OH	OH

\* The formula for bile acids shown in Table II has a dotted line to represent the bond between C-17 and C-20 representing the side chain in the *trans* position to the C-19 methyl group and below the plane of the ring. This convention has been adopted in a number of papers published recently in the *Helvetica Chimica Acta* both on bile acids and cholesterol derivatives. In this review the older convention of a solid line is used without implying any particular steric relationship in the attachment of the side chain. (Cf. references 149 and 150.)

TABLE III

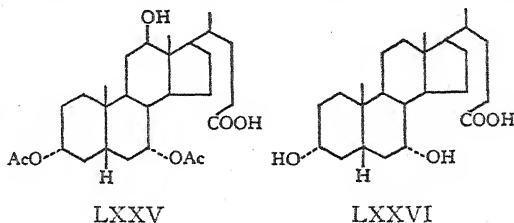
## ROTATION AND STRUCTURE (46)\*

	$[\alpha]_D$	$[M]_D$	$\Delta[M]_D^\dagger$
Lithocholic acid			
[3( $\alpha$ )-hydroxycholanolic acid] .....	+32.1°	+121°	....
Chenodesoxycholic acid			
[3( $\alpha$ ),7( $\alpha$ )-dihydroxycholanolic acid] .....	+12.5°	+ 49°	- 72°
Ursodesoxycholic acid			
[3( $\alpha$ ),7( $\beta$ )-dihydroxycholanolic acid] .....	+57.0°	+224°	+103°
Desoxycholic acid			
[3( $\alpha$ ),12( $\beta$ )-dihydroxycholanolic acid] .....	+57.0°	+224°	+103°
Cholic acid			
[3( $\alpha$ ),7( $\alpha$ ),12( $\beta$ )-trihydroxycholanolic acid] ....	+36.9°	+149°	+ 28°

\* All rotations in alcohol. † Difference in molecular rotation compared to lithocholic acid.

The transformation of cholic acid to chenodesoxycholic acid by a new route which does not alter the 7-hydroxyl group confirmed the earlier (48) assigned steric relationships for these bile acids. Cholic acid was transformed to 3( $\alpha$ ),7( $\alpha$ )-diacetoxy-12( $\beta$ )-hydroxycholanolic acid (49) (LXXV) which was then methylated, oxidized to the

ketone, and converted to chenodesoxycholic acid (LXXVI) [3( $\alpha$ ),-7( $\alpha$ )-dihydroxycholanolic acid] by a Wolff-Kishner reduction (46).



An improved laboratory preparation of nordesoxycholic acid from desoxycholic acid through the intermediary 3,12-diacetoxybisanthracene has been described (50). The stepwise bacterial oxidation of cholic acid through the action of *Alcaligenes faecalis* (51) probably follows the same course as that observed in the chromic acid oxidation (52, 53). The order of oxidation of the hydroxyls is 7, 12, 3; the first product is 3,12-dihydroxy-7-ketocholanolic acid; the second is 3-hydroxy-7,12-diketocholanolic acid; and the final product is 3,7,12-triketocholanolic acid (dehydrocholic acid).

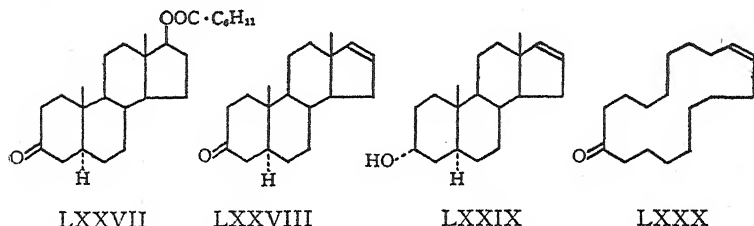
The specificity of the Pettenkofer reaction for steroids, particularly for bile acids, has been qualitatively investigated, and it was found that all steroids having a hydroxyl at C-7 or a group easily changed to a hydroxyl gave a positive test. Dehydro-*trans*-androsterone acetate also gave a strong reaction which might be utilized in detecting this compound in hormone mixtures (54).

A photometric method (55, 56) for the determination of cholates in blood and bile has been adapted for the photoelectric filter photometer, and it has been shown that cholates and their conjugates can be determined with accuracy even in the presence of mono- and dihydroxycholates (57).

#### STERIOD HORMONES AND RELATED COMPOUNDS

In order to increase the water solubility of the relatively water-insoluble steroid hormones a large number of saccharides have been prepared from those compounds having a free hydroxyl group. This has been accomplished by the action of the appropriate acetobromo sugar in the presence of silver carbonate. Particular emphasis has been placed on the preparation of the saccharides of desoxycorticosterone, the maltoside of which forms a stable 10 per cent solution in water (58, 59).

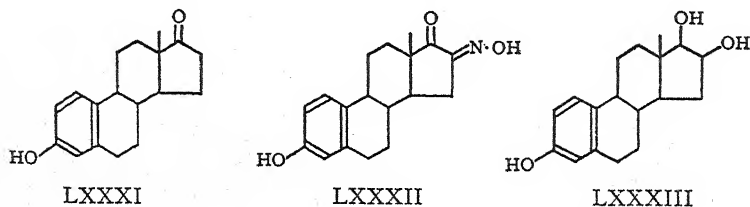
The synthesis of  $\Delta^{16}$ -androst-3( $\alpha$ )-ol (LXXIX) and its  $\beta$ -epimer was accomplished by the thermal cleavage of the hexahydrobenzoate of androstane-17( $\beta$ )-ol-3-one (LXXVII) to form  $\Delta^{16}$ -androst-3-one (LXXVIII) from which by a Meerwein-Ponndorf reduction the epimeric  $\alpha$ - and  $\beta$ - $\Delta^{16}$ -androstenols were obtained and separated with digitonin. The epimeric androstenols were identical with two com-



pounds having a musk-like odor isolated from swine testes (117). For comparison, the structure of civetone (LXXX), a constituent of natural musk, is written in a steroid pattern (60).

Of interest in connection with the chemistry of saponins reviewed elsewhere in this volume is the continuation of the work on the reaction of acetylene and propargyl alcohol with androstane-3( $\beta$ )-ol-17-one and subsequent transformation products (61, 62). Mention should also be made of the synthesis of a physiologically active 10-norprogesterone and 10-nor-11-desoxycorticosterone acetate from strophanthin (63, 64).

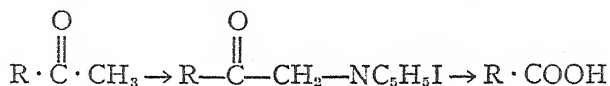
Four isomeric 3,16,17-estriols (LXXXIII) are theoretically possible. The only previously known estriol is the naturally occurring hormone theelol. A second isomer designated as isoestriol-A has been synthesized from estrone (LXXXI) by benzylation followed by formation of the 16-oximino derivative (LXXXII), saponification of the ester, reductive hydrolysis of the oximino group with zinc in acetic acid, and finally hydrogenation of the ketol. The steric relationships of the hydroxyls at 16 and 17 are as yet undetermined (65).



The first purely chemical transformation of estrone to the estriol, theelol, has been announced without information as to the route followed (66).

Pregnane-3( $\alpha$ )-ol-20-one has been isomerized at C-17 to 17-isopregnane-3( $\alpha$ )-ol-20-one with alkali, a reaction characteristic of other known 20-ketones due to enolization (67).

The degradation of  $\Delta^5$ -3( $\beta$ )-hydroxypregnene-20-one to  $\Delta^5$ -3( $\beta$ )-hydroxyetiocholenic acid was accomplished by treatment with iodine and pyridine. A pyridinium iodide was formed which was decomposed with alkali to give the acid (68) as follows:



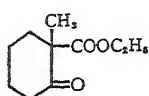
Irradiation of estrone (LXXXI) with ultraviolet light gave an isomeric lumiestrone, probably 13-epiestrone by inversion of the angular methyl group (69).

## NUCLEAR SYNTHESSES

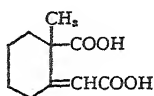
Of interest in connection with the stereochemical configuration of the C and D rings is the synthesis of both the *cis* and *trans* forms of 8-methyl-1-hydrindanone (XCI). The *cis* form, previously prepared by a number of investigators, had earlier been assigned the *trans* configuration but recently was tentatively placed in the *cis* category (70). This *cis* form was prepared by the same procedures earlier employed in the synthesis of equilenin. A mixture of isomeric acids, LXXXV and LXXXVI (possibly both geometrical isomers of LXXXV), was obtained by the Reformatsky reaction of 2-methyl-2-carboethoxycyclohexanone (LXXXIV) with ethyl bromoacetate followed by dehydration and hydrolysis. Both acids gave a mixture of *cis* and *trans* 2-methyl-2-carboxycyclohexane-1-acetic acids (LXXXVII) on reduction. In this mixture the *cis* form predominated which was converted to the dimethyl ester of 2-methyl-2-carboxycyclohexyl propionic acid (LXXXIX) by means of the Arndt-Eistert reaction on the corresponding acid chlorides obtained from the methylation of LXXXVII followed by selective hydrolysis to the monomethyl ester (LXXXVIII). The ester LXXXIX was cyclized with sodium methoxide, hydrolyzed, and decarboxylated to give the desired *cis*-8-methyl-1-hydrindanone (XCI). *Trans*-8-methyl-1-hydrindanone (XCI) was synthesized from the readily available 1-methyl-



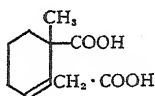
2-cyclohexene-1,2-dicarboxylic acid (XC) which was reduced and converted to the *trans* form of LXXXVII. By the same method it was transformed to the desired *trans*-8-methyl-1-hydrindanone (XCI) (71).



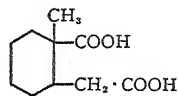
LXXXIV



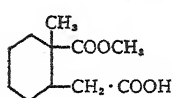
LXXXV



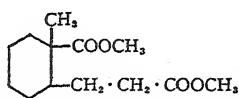
LXXXVI



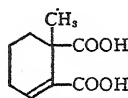
LXXXVII



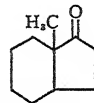
LXXXVIII



LXXXIX

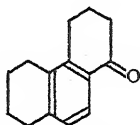


XC

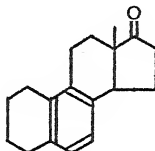


XCI

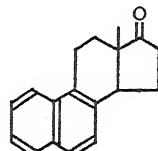
The *cis* and *trans* forms of 17-equilenone (XCIV) (desoxyequilenin), designated as  $\alpha$ - and  $\beta$ - due to uncertainty as to their configuration, were prepared by dehydrogenation with sulphur of the corresponding  $\alpha$ - and  $\beta$ -1,2,3,4-tetrahydro-17-equilenones (XCIII) synthesized from 1-keto-*sym*-octahydrophenanthrene (XCII) by the methods previously employed for the synthesis of equilenin (72). One of the optically active forms of 1,2,3,4-tetrahydro-17-equilenone (XCIII) had previously been prepared from equilenine (73).



XCII

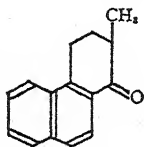


XCIII

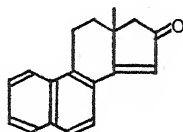


XCIV

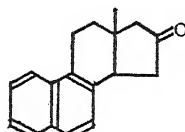
Progress in the total synthesis of steroids related to the female sex hormones has been reported with the synthesis of 16-equilenone (XCVII) starting with 1-keto-2-methyltetrahydrophenanthrene (XCV) through the unsaturated ketone (XCVI). Due to the double



XCV



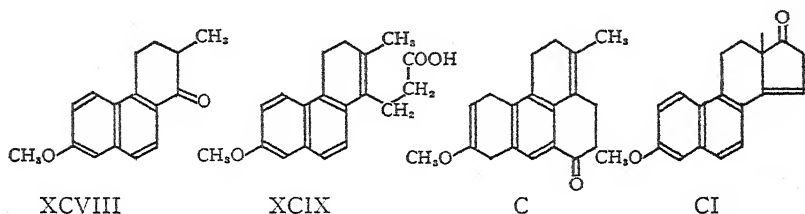
XCVI



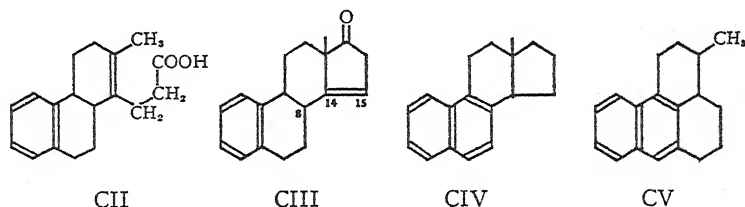
XCVII

bond blocking position 15 in ring D, this ketone is of importance in condensation reactions leading to the introduction of groups in the 17 position. The corresponding 16,17-diketone was also prepared, but so far reduction failed to give the desoxyequilenin analogue of estriol (74).

The Reformatsky reaction of  $\beta$ -bromopropionic acid with 1-keto-2-methyl-1,2,3,4-tetrahydro-7-methoxyphenanthrene (XCVIII) gives an unsaturated acid (XCIX) whose double bond is probably in the ring instead of the propionic acid side chain. On ring closure a benzanthrane ketone (C) instead of the desired cyclopentanophenanthrene derivative (CI) was obtained. The hydrogenated acid from XCIX also gave a benzanthrane ketone (75).

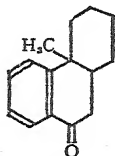


This failure to obtain the cyclopentanophenanthrene derivatives mentioned was paralleled in the attempted ring closure of the  $\beta$ -[2-methyl-3,4,9,10,11,12-hexahydro-phenanthryl-(1)]-propionic acid (CII) to give the ketone (CIII) (or with the double bond at 8:14 instead of 14:15). The Clemmensen reduction of the ketone actually obtained followed by dehydrogenation gave a liquid hydrocarbon not identical to the known equilenane (CIV) and probably produced the benzanthrane structure (CV) (76).

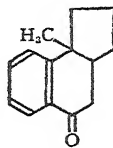


Several hydroaromatic compounds containing angular methyl groups have been prepared with a view to testing their physiological activities. The ketohydrophenanthrene (CVI) and a ketocyclopentano-

hydronaphthalene (CVII) and the corresponding hydrocarbons have been synthesized (77).



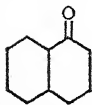
CVI



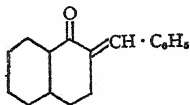
CVII

A number of earlier papers on the synthesis of compounds related to vitamin D have been withdrawn pending verification of experimental work (78).

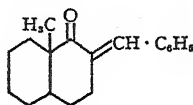
The stepwise synthesis of the steroid nucleus with its angular methyl groups ring by ring is a task of considerable difficulty and methods for the building up of this skeleton involving the condensation of two preformed ring systems offer very attractive possibilities. Methods for the direct introduction of an angular methyl group into such ring systems are essential to the success of such an approach. The early attempts to accomplish this have either given negative results or the yields have been poor except for a method applicable to phenolic tetrahydronaphthalenes (79). Recently a method giving excellent yields has been developed as a model, starting with decalone-1 (CVIII). The decalone was condensed with benzaldehyde to give *trans*-2-benzaldecalone-1 (CIX) which was methylated with methyl iodide in the presence of potassium *t*-butoxide to give a mixture of *cis*- and *trans*-2-benzal-9-methyldecalone-1 (CX). Separation of the isomers was accomplished by fractional crystallization and by utilizing the difference in rates of formation of the semicarbazones. Chlorination followed by alkaline hydrolysis gave the *cis* and *trans* isomers of 9-methyldecalone-1 (CXI) (80).



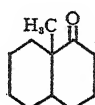
CVIII



CIX



CX

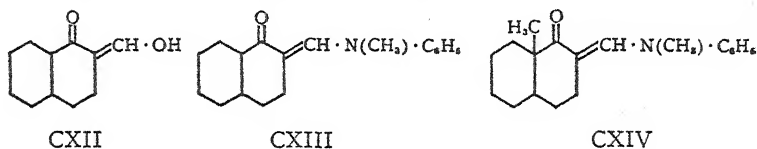


CXI

The procedure described depends upon the blocking of the carbon atom adjacent to the keto group. Previous attempts at direct methylation resulted in the formation mainly of the  $\alpha$ -methyl derivative accom-

panied by only small amounts of the angular methylated compound (81) with two exceptions (82).

A new procedure which has the advantage of not requiring a chlorination for the removal of the blocking group has also been developed. *Trans*-decalone-1 (CVIII) and ethyl formate were condensed in the presence of sodium to give 2-formyldecalone-1 (CXII) which with methylaniline gave 2-methylanilinomethylene decalone-1 (CXIII). Methylation with methyl iodide and sodamide gave a compound (CIV) which on hydrolysis with dilute hydrochloric acid gave the formyl derivative. Treatment with dilute alkali yielded a mixture of *cis*- and *trans*-9-methyldecalone-1 (CXI) separable by fractional hydrolysis of the semicarbazones (83).



Exploratory work on the synthesis of the sterol nucleus by the coupling of two ring systems followed by the introduction of the angular methyl groups has been reported but the goal has not yet been attained (84). An excellent review on steroid syntheses covering the work from 1939 to 1943 is now available (85).

## METABOLISM OF STEROIDS

### CHOLESTEROL

Although the role of cholesterol as an intermediary in the formation of the steroid compounds, such as the adrenal and sex hormones, bile acids, and vitamin D, has long been postulated, it has only been in the past few years that direct evidence in support of this hypothesis has been established. Perhaps the most convincing proof is that resulting from the use of cholesterol containing deuterium as an indicator. When deuteriocholesterol was fed to dogs, the cholic acid excreted in the bile contained deuterium (89). It has now been shown that on feeding deuteriocholesterol to a woman in the eighth month of pregnancy the pregnanediol glucuronidate isolated from the urine contained a proportion of deuterium such that one is forced to the conclusion that the major part of the pregnanediol formed was derived from cholesterol (90). Although pregnanediol is not, *per se*, a hormone, it

is a product of progesterone metabolism, and the inference is clear that, at least under the conditions of the experiment, progesterone is largely formed directly from cholesterol. Thus on the basis of these two experiments, it has been demonstrated that degradation of the side chain of cholesterol occurs in the body with the formation of a bile acid and a derivative of progesterone. Furthermore, the recovery of deuteriocholic acid is evidence for the oxidation of the cholesterol nucleus in the body at positions 7 and 12 and, in addition, for the conversion of a cholesterol to a coprosterol-type derivative. Likewise, the pregnanediol experiment can be interpreted as evidence for a mechanism whereby a cholesterol-type derivative is converted first to the  $\alpha$ : $\beta$  unsaturated ketone of the progesterone type and then is reduced to a pregnane compound with the 3-hydroxyl having the  $\alpha$ , rather than  $\beta$ , configuration of the original derivative.

Succinylsulfathiazole, which exerts a bacteriostatic action on *Esch. coli*, and carbarsone, which frees rats from intestinal amoebae, both completely inhibit the formation of coprosterol in the rat intestine. Since neither cholesterol nor cholestenone incubated with a mixed culture of intestinal bacteria gave rise to coprosterol, the conclusion was drawn that it was improbable that intestinal bacteria played any role in the formation of coprosterol. Indirect evidence led to the same conclusion with regard to intestinal protozoa. The inhibitory action on coprosterol formation of succinylsulfathiazole and carbazone was, therefore, believed to be unconnected with their action on intestinal microorganisms (88, 91). It had earlier been shown with deuterium-labeled cholestenone that this compound could be transformed to coprosterol in the dog (92). The isolation of cholestenone from pig testes (93) and evidence for its presence in pig spleen (94) might be interpreted in favor of the view that the conversion of cholesterol to coprosterol does not necessarily take place entirely in the intestine. The two-stage mechanism of this conversion (87), in which cholestenone is considered to be an intermediate, was supported by the isolation of cholestenone from rat feces (88).

The isolation of steroids, particularly  $C_{27}$  cholesterol derivatives, from various organ extracts has been continued. The unsaponifiable fraction from the acetone extracts of human aortas has been reinvestigated and, in addition to the earlier isolated cholesterol and cholestanol, the presence of much smaller amounts of  $\Delta^{3,5}$ -cholestadiene-7-one (CXXIII),  $\Delta^{4,6}$ -cholestadiene-3-one (CXXII), cholestane-3( $\beta$ ),5,6-*trans*-triol (CXX), and 7( $\beta$ )-hydroxycholesterol (CXVII), together

with batyl alcohol and four unidentified constituents, has been reported (95). The  $C_{19}$  and  $C_{21}$  steroids are conspicuous by their absence. It is possible that the relatively large amount of  $\Delta^{3,5}$ -cholestadiene-7-one (CXXIII) found is formed by dehydration during the extraction procedure from 7-ketocholesterol (CXXV), the presence of which in this material had earlier been suspected on the basis of spectroscopic evidence (96, 97).

The unsaponifiable fraction from 1500 kg. of fresh hog spleen was found to contain amounts, ranging from a few milligrams to several grams, of  $\Delta^5$ -cholestene-3( $\beta$ ),7( $\alpha$ )-diol (CXVI),  $\Delta^4$ -cholestene-3( $\beta$ ),6-diol (CXV), cholestane-3( $\beta$ )-ol-6-one (CXXIV),  $\Delta^{3,5}$ -cholestadiene-7-one (CXXIII),  $\Delta^{4,6}$ -cholestadiene-3-one (CXXII), and a compound (CXIX)  $C_{27}H_{46}O_2$ , m.p. 155–56° C., which is apparently stereoisomeric with the  $\Delta^6$ -cholestene-3,5-diol (CXVIII) (Compound "A") formed by the allylic rearrangement in the presence of acids of  $\Delta^5$ -cholestene-3( $\beta$ ),7( $\beta$ )-diol (CXVII) (98). In addition, batyl alcohol, palmitylsphingosine (?), and several unidentified compounds were isolated (94). Table IV, summarizing the  $C_{27}$  transformation products of cholesterol, is adapted from this paper (94) with the addition of  $\beta$ -cholestanol [cholestane-3( $\beta$ )-ol] (CXXVI) and 7-dehydrocholesterol [ $\Delta^{5,7}$ -cholestadiene-3( $\beta$ )-ol] (CXXVII) (99, 100). A tabulation of cholesterol transformation products with literature references may also be found in a short review on the metabolism of cholesterol (101). It was pointed out (94) that all of the products formed from the oxidation of colloidal cholesterol solutions with air or oxygen (102, 151), together with those formed in the oxidation of cholesterol in the presence of ultraviolet light (103), have been isolated from organ extracts. The only exception is the unidentified diol, m.p. 177° C., obtained by the photooxidation. Compounds isolated from organ extracts and not from the *in vitro* oxidations are cholestane-3( $\beta$ ),5,6-*trans*-triol (CXX),  $\Delta^4$ -cholestene-3-one (CXXI),  $\Delta^{4,6}$ -cholestadiene-3-one (CXXII), cholestane-3( $\beta$ )-ol-6-one (CXXIV), and  $\Delta^6$ -cholestene-3,5-diol (CXIX), m.p. 155–56° C.

In the isolation of these products from organ extracts, the possibility that a part, or all, of a particular compound might arise from oxidation during the course of the saponification and extraction cannot be completely excluded without a great deal of further investigation. The problem is one of great technical difficulty due to the demonstrated sensitivity of colloidal cholesterol solutions to oxidation and also the proportionately minute amounts of the products isolated from

TABLE IV  
C<sub>27</sub> TRANSFORMATION PRODUCTS OF CHOLESTEROL

Compound	Formula	Cholesterol		Organ Extracts					
		Auto-oxid.	Photo-oxid.	Mare or Human Serum	Animal Livers	Pig Testes	Human Aorta	Pig Spleen	Pig Skin
1. $\Delta^4$ -Cholestene-3( $\beta$ ),6-diol	CXV	..	+	..	..	..	..	+	..
2. $\Delta^5$ -Cholestene-3( $\beta$ ),7( $\alpha$ )-diol	CXVI	+	+	+	+	..	..	+	..
3. $\Delta^5$ -Cholestene-3( $\beta$ ),7( $\beta$ )-diol	CXVII	..*	..	+	+	..	+	..*	..
4. $\Delta^6$ -Cholestene-3,5-diol, m.p. 140°	CXVIII	+	..	..	..	..	..	..	..
5. $\Delta^6$ -Cholestene-3,5-diol, m.p. 156°	CXIX	..	..	..	..	..	..	..†	..
6. Diol C <sub>27</sub> H <sub>46</sub> O <sub>2</sub> , m.p. 177°	§	..	+	..	..	..	..	..	..
7. Cholestane-3( $\beta$ ),5,6- <i>trans</i> -triol	CXX	..	..	..	+	+	+	..	..
8. $\Delta^4$ -Cholestene-3-one	CXXI	..	..	..	..	+	..	?	..
9. $\Delta^4$ , $\Delta^6$ -Cholestadiene-3-one	CXXII	..	..	..	..	..	+	+	..
10. $\Delta^3$ , $\Delta^5$ -Cholestadiene-7-one	CXXIII	+	..	..	..	+	+	+	..
1. Cholestane-3( $\beta$ )-ol-6-one	CXXIV	..	..	..	..	..	..	+	..
2. $\Delta^5$ -Cholestene-3( $\beta$ )-ol-7-one	CXXV	+	..	++	..	+	..†	..†	..
3. Cholestane-3( $\beta$ )-ol	CXXVI	..	..	+	..	..	+	..	..
4. $\Delta^5$ ,7-Cholestadiene-3( $\beta$ )-ol	CXXVII	..	..	..	..	..	..	..	+†

\* Not isolated, but probably converted to CXVIII.

† Not isolated, but probably converted to CXXIII.

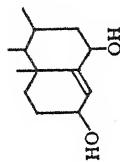
‡ Stereoisomeric with autooxidation diol ( $\Delta^6$ -cholestene-3,5-diol) CXVIII; structure not certain.

§ Structure not known.

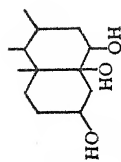
|| Unpublished work referred to in subsequent paper (148, 102).

¶ The presence of 7-dehydrocholesterol in much lower concentrations in other tissues has been demonstrated spectroscopically and by conversion to vitamin D<sub>3</sub>.

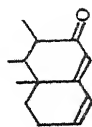
FORMULAE FOR TABLE IV\*



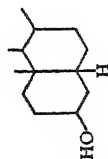
CXV.  $\Delta^4$ -Cholestene-3( $\beta$ ),6-diol



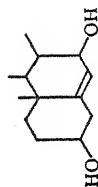
CXX. Cholestane-3( $\beta$ ),5,6-*trans*-triol



CXXIII.  $\Delta^{3,5}$ -Cholestadiene-7-one

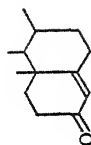


CXXXVI. Cholestane-3( $\beta$ )-ol  
( $\beta$ -Cholesterol)

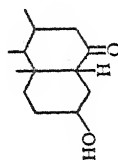


CXVI.  $\Delta^5$ -Cholestene-3( $\beta$ ),7( $\alpha$ )-diol  
[7( $\alpha$ )-Hydroxycholesterol]

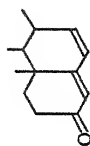
CXVII.  $\Delta^5$ -Cholestene-3( $\beta$ ),7( $\beta$ )-diol  
[7( $\beta$ )-Hydroxycholesterol]



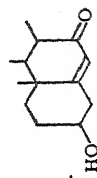
CXXI.  $\Delta^4$ -Cholestene-3-one  
(Cholestenone)



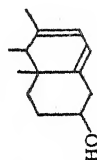
CXXIV. Cholestane-3( $\beta$ )-ol-6-one



CXXII.  $\Delta^{4,6}$ -Cholestadiene-3-one



CXXV.  $\Delta^5$ -Cholestene-3( $\beta$ )-ol-7-one  
(7-Ketocholesterol)



CXXVII.  $\Delta^{5,7}$ -Cholestadiene-3( $\beta$ )-ol  
(7-Dehydrocholesterol)

\* The formulae in this table do not depict steric relationships.



animal organs. However, the products listed in Table IV seem likely to be present either in the organs themselves, or formed as the result of simple dehydrations, or rearrangements of their mother substances. For example, the  $\Delta^{3,5}$ -cholestadiene-7-one (CXXIII) probably arises from the dehydration of  $\Delta^5$ -cholestene-3( $\beta$ )-ol-7-one (CXXV).

A number of unidentified products and pregnane-3( $\beta$ ),20( $\alpha$ )-diol have been isolated from the neutral nonsaponifiable fraction of ox bile (115). No hydroxycholesterol could be isolated from extracts of human and dog adrenals (116).

Besides the cholesterol transformation products previously discussed, swine testes have yielded two steroids with a musk-like odor identified as  $\Delta^{16}$ -androstene-3( $\alpha$ )-ol (LXXIX) and  $\Delta^{16}$ -androstene-3( $\beta$ )-ol (117); also isolated was chimyl alcohol (*d*- $\alpha$ -hexadecylglyceryl ether), which was shown to be identical with the "testriol" earlier obtained from the same source (118).

The possible role played by 7-hydroxycholesterol and 7-dehydrocholesterol in the animal organism, apart from their being likely intermediates in the biogenesis of vitamin D, has not been investigated. For example, no explanation has been found for the relatively high concentration of 7-dehydro sterols found in certain invertebrates.

The subcutaneous injection or feeding of 7-hydroxycholesterol, 7-dehydrocholesterol, and 7-ketocholesterol to rats on a special deficient diet prevented rickets in animals subjected to ultraviolet radiation (104). The remarkable finding that 7-hydroxycholesterol was substantially more active than 7-dehydrocholesterol needs further experimental investigation. The fact that 7-hydroxycholesterol has been shown to act as a true provitamin D in the animal organism leads one to consider the possibility that 7-dehydrocholesterol may to some extent be a detoxification or end metabolic product of cholesterol oxidation. It is interesting to note that ergosterol is formed by yeast to a much greater extent than normal under conditions of rapid growth with oxygenation (105) and that several species of *Penicillium* form ergosterol in surface cultures, but not in submerged fermentations (106, 107).

The cholesterol metabolism of the adrenal gland in relation to the thyroid hormone has been further studied from the standpoint of the varying cholesterol content of the gland, and the recent literature has been reviewed (108, 109).

It has been pointed out that the enzyme systems which might alter the course of cholesterol transformation have not yet been adequately

explored (101). The absence of  $C_{18}$  and  $C_{21}$  steroids in pig spleen would seem to indicate a specific side-chain degrading enzyme system in the adrenal cortex which is absent in the spleen. The relatively high concentration of 7-dehydrocholesterol in the skin, as compared to other organs, seems to point to the presence in it of a specific enzyme system (99, 100).

In connection with the broad problem of cholesterol metabolism, earlier work on the role of sterols (110) on insect nutrition has been extended, and it has been found that the larvae of *Dermestes vulpinus* grown on a "synthetic" diet with yeast cannot utilize plant sterols, such as sitosterol, ergosterol, and vitamin  $D_2$ , but require cholesterol or 7-dehydrocholesterol. The latter is much more effective in promoting growth than the former (111, 112).

The importance of slight changes in the steroid side chain on the physiological effect of the sterols recalls a similar effect found in the species specificity of the antirachitic action of various vitamins D which differ only in the side chain.

With regard to the synthesis of cholesterol in the animal body, recent studies carried out with the aid of deuterium confirm the view that acetic acid is a specific precursor of cholesterol. Cholesterol isolated from mice and rats fed deuterioleucine and deuterioisovaleric acids contained deuterium, whereas none was found in similar experiments with valine and isobutyric acid. In all cases, deuteriocholesterol was formed only from compounds which also gave rise to deuterioacetic acid (113, 114).

#### STEROID HORMONES

The work on the various aspects of the metabolism of the steroid hormones has in recent years become so voluminous that the subject as a whole seems best treated in separate periodic reviews, as, for example, the "Intermediate Metabolism of Sex Hormones" (119), "Hormones of the Adrenal Cortex" (120), "The Physiological and Clinical Significance of the Urinary 17-Keto Steroids" (121), "The Role of the Adrenal Cortex in Physiological Processes" (122), and "Hormones of the Adrenal Cortex" (86).

Rapid progress has been made in the isolation and estimation of the urinary steroids resulting from the metabolism of the steroid hormones, particularly the 17-keto steroids fraction derived from the adrenal cortical hormones. Because of its clinical significance and re-

lationship to hormones, this material is reviewed in this volume, page 561.

The isolation of dehydroisoandrosterone sulphate from normal male urine has been reported (123). An androstenetriol obtained from normal human urine is now believed to be identical with androstene-3( $\beta$ ),16,17-triol, shown to have been present in the urine of a boy with adenocarcinoma of the adrenal cortex (124, 125).

The conversion of desoxycorticosterone to pregnane-3( $\alpha$ ),20( $\alpha$ )-diol in man and also in a normal and an ovariectomized chimpanzee has been demonstrated. This is the first case in which a hydroxyl group in the side chain has been replaced by hydrogen (126, 127). The oral administration of testosterone propionate to adult male chimpanzees resulted in a thirteenfold increase in urinary androgenic activity, of which 31.3 per cent was accounted for by androsterone, 6.2 per cent by 17-androstenone; etiocholane-3( $\alpha$ )-ol-17-one was also isolated (128). The conversion of testosterone to androsterone by the pregnant rhesus monkey was demonstrated by the isolation of the latter in the urine, whereas the urine of pregnant control monkeys not given testosterone failed to show the presence of androsterone (129).

The cortin-like substances extractable from human and monkey urines appear definitely to originate in the adrenals. In monkeys, gonadectomy does not affect the excretion of the material, but none is found after adrenalectomy, unless given adrenal cortical extracts. Administration of desoxycorticosterone in only a single isolated instance caused the appearance of the cortin-like substance in the urine of a monkey. Patients with adrenal insufficiency, who responded to cortical extracts with a significant increase in the urine of the cortin-like substance, failed to do so when given desoxycorticosterone (130, 131). Urines collected after the stress of surgery contained up to thirty times the normal amount of this material, which indicates that the probable source of the activity is in the adrenal cortex (132). In this connection it is interesting to note that the administration of synthetic  $\Delta^5$ -pregnenolone combats fatigue, probably due to its sparing action on the metabolism of the adrenal cortical hormones (133, 134).

The isolation of etiocholane-3( $\alpha$ ),12( $\beta$ )-diol-17-one has been reported from extracts of the adrenal cortex (135). Hepatectomy increased the sensitivity of rats to the anesthetic action of steroids. This indicates the liver as a site of steroid detoxification (136).  $\Delta^4$ -Androstene-3,17-dione incubated with rabbit liver slices was transformed to

testosterone with small amounts of *cis*-testosterone and hydroxylated 17-ketosteroids (137).

The inactivation of estradiol by rat liver slices *in vitro* did not occur when the livers were obtained from animals maintained on riboflavin and thiamine deficient diets. Diets deficient in pyridoxine, pantothenic acid, biotin, and vitamin A had no effect (138). Similar conclusions based on the ability of estrone and  $\alpha$ -estradiol to produce estrus in rats on thiamine and riboflavin deficient diets were reached. In addition to the other B-complex vitamins mentioned, choline chloride had no effect (139).

A study of the metabolism of estrone in normal and partially hepatectomized rats indicated that the conversion of estrone to estriol was most markedly interfered with by liver lobe extirpation because the strong phenolic estrogen in the urine increased a little over two times normal, the weak phenolic nonketonic estrogen four times, and the weak phenolic ketonic estrogen nearly six times (140). The intra-splenic injection of estrogens and their esters in rats with and without spleen transplants strengthened the evidence that the liver is the site of estrogen inactivation (141). The excretion of estrogenic material in the urine of ovariectomized mice having spontaneous adrenal tumors was found to be four times that of tumor-free normal controls from the same strain (142).

A curious observation worth reinvestigating in view of recent advances in cholesterol oxidation and metabolism is the reported estrogenic action of an extract of the sun-dried skin of various fishes. Extracts of fresh, unexposed skin, muscle, or viscera of the fish lacked estrogenic activity (143). The elevation of uterine  $\beta$ -glucuronidate activity by estrogenic hormones has been reported (144). Following the oral administration of anhydroxyprogesterone and progesterone an increase was found in the excretion of sodium pregnanediol glucuronidate in the urine of patients with secondary amenorrhea.

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# THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR

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Under war conditions, which have continued to prevail since this topic was last reviewed,<sup>1</sup> even English and American scientific periodicals usually require several months to reach Australia. Moreover, the slow mail schedules have necessitated an early dispatch of the typescript of this review. Consequently it has not been possible to incorporate material published overseas much later than the middle of 1944. It has been necessary, too, to rely occasionally upon abstracting journals. In particular, and as the matters can be embraced in other sections of this volume, the sulfur-containing vitamins and chemotherapeutic agents have been excluded.

## CHEMICAL ASPECTS

*Syntheses.*—A cystine tripeptide containing two double bonds and a cystine pentapeptide containing four double bonds, have been synthesized (1). The former, bis(acetyldehydrophenylalanyl)-*l*-cystine (m.p. 212–13° C., decomp.), was prepared by condensing cystine with acetyldehydrophenylalanyl azlactone; the latter, bis(acetyldehydrophenylalanyldéhydrophenylalanyl)-*l*-cystine (m.p. 209–11° C.), was prepared by an analogous azlactone method.

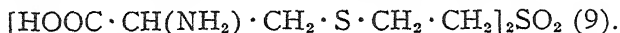
*dl*-Methionine containing an excess of the stable isotopes C<sup>13</sup> (in the β and γ positions) and S<sup>34</sup>, has been prepared from sodium cyanide containing excess of C<sup>13</sup> and sodium sulfite containing excess of S<sup>34</sup> (2). It has been shown (3) that the so-called "pseudo-methionine" contaminant of *dl*-methionine obtained by the large-scale procedure of Barger & Weichselbaum (4), is a mixture of the *dl*- and *meso*- forms of ω,ω'-bimethionine, [HOOC · CH(NH<sub>2</sub>) · CH<sub>2</sub> · CH<sub>2</sub> · S · CH<sub>2</sub>]<sub>2</sub>.

Ethionine, the ethyl homologue of methionine, has been synthesized (5) from ethyl β-chloroethyl sulfide and diethyl phthalimidosodium-alonate as starting materials, the principles of synthesis being essentially those of Barger & Weichselbaum (4) for the synthesis of methionine. Lanthionine has been synthesized from dichlorodimethyl

<sup>1</sup> Andrews, J. C., *Ann. Rev. Biochem.*, 12, 115–34 (1943).

sulfide and diethyl phthalimidosodi-malonate in a somewhat similar way (6).

The synthesis of *dl*-methionine phenyl hydantoin (m.p. 109–10° C) from methionine and phenylisocyanate has been described (7). Methionine-*N-p*-phenylazobenzoyl methyl ester has been prepared from the hydrochloride of the methyl ester of methionine and phenylazobenzoyl chloride (8).  $\beta,\beta'$ -Dichlorodiethyl sulfone reacts with cysteine in neutral solution to form *S,S'*-dicysteinyl diethyl sulfone,



An interesting synthesis of taurine has been described (10).  $\beta$ -Aminoethyl hydrogen sulfate, from ethanolamine and fuming sulfuric acid, is made to react with sodium sulfite, taurine resulting from an unusual rearrangement in which a carbon-oxygen link is replaced by a carbon-sulfur link.

In connection with their mercapturic acid studies, Zbarsky & Young (11) have synthesized phenyl-*l*-cysteine and *l*-phenyl-mercapturic acid. The former was prepared by two methods: (a) by decomposition of the product of interaction of cuprous cysteine mercaptide and diazotized aniline by heating it in solution at 60 to 70° C., and (b) by debromination of *p*-bromophenyl-*l*-cysteine with sodium amalgam. The *l*-phenylmercapturic acid was synthesized in three ways: (a) by decomposition of the product of interaction of acetylcysteine and diazotized aniline, (b) by acetylation of phenyl-*l*-cysteine, and (c) by debromination of *p*-bromophenylmercapturic acid, the bromo derivative itself being readily formed by biological synthesis. *p*-Fluorophenyl-*l*-cysteine has been synthesized (12) by treating cuprous cysteine mercaptide with diazotized *p*-fluoroaniline. *In vitro* acetylation of the substance to give *p*-fluorophenylmercapturic acid was achieved with acetic anhydride and sodium hydroxide (12).

*Sulfur in proteins.*—Virtually all the sulfur in the pituitary lactogenic hormone (13) is attributable to cystine (3.11 per cent) and methionine (4.31 per cent). Earlier estimates (14, 15) of the cystine content are 3.0 and 3.36 per cent, respectively.

The cysteine, cystine, and methionine contents of various proteins have been estimated (16) following acid hydrolysis under nitrogen. It was found that the cysteine values agreed fairly well with the estimated sulphydryl groups in the unhydrolysed proteins. Only 82 per cent of the sulfur in edestin was identified.

The rib-grass strain of tobacco mosaic virus has been found (17)

to contain 0.68 per cent of cysteine and about 2 per cent of methionine, the total sulfur content being about 0.62 per cent. The sulfur distribution in tobacco mosaic virus is so different from this that the taxonomic distinction conveyed by "rib-grass strain" seems inadequate. The cystine content of the protein of the virus of grasserie is reported to be 0.52 per cent (18).

A further contribution to the controversy concerning the possibility of variation in the composition of the whole serum proteins with diet has been made (19). No significant differences were found with dogs. Nitrogen, sulfur, and cystine contents ranging from 11.55 to 14.35 per cent, from 1.09 to 1.38 per cent, and from 3.06 to 3.31 per cent, respectively were reported. On the other hand, in continuation of earlier studies (20) Vassel *et al.* (21) claim that in addition to inversion of the serum albumin-globulin ratio in dogs infected with Type I pneumococcus, there is a reduction in the cystine content of the albumin fraction. Although the cystine content of the globulin fraction was considered not to have changed, the variation from animal to animal was rather large. Incidentally, the effects of inanition and of ingestion of the hepatotoxic agents, hydrazine and phosphorus, upon the partial compositions, including sulfur and cystine contents, of tissue proteins of rabbits were found to be insignificant (22).

The total sulfur and cystine contents of  $\beta$ -lactoglobulin estimated by Brand & Kassell (23) have been satisfactorily confirmed by Bolling & Block (24) who found 1.68 per cent of sulfur and 3.48 per cent of cystine in a sample containing 15.53 per cent of nitrogen. The whole proteins in the main photosynthesizing tissues of one bryophyte and two pteridophytes were found (25) to contain, in terms of the protein nitrogen, 1.14 to 1.31 per cent of cyst(e)ine and 1.36 to 1.63 per cent of methionine, these values being similar to those found for phanerogams. Only a trace of sulfur was found in the unautolyzable protein of the mold, *Aspergillus sydowii* (26).

Block & Bolling (27) have published a table of amino acid (including cystine and methionine) contents of proteins important in the nutrition of man and other animals. They have drawn attention to the high methionine content of corn gluten (5.5 per cent, assuming 16 per cent of nitrogen in the protein). Part of the pronounced variation in cystine content (0.42 to 1.42 per cent of the nitrogen) and methionine content (1.80 to 4.11 per cent of the nitrogen) of the whole proteins in the muscles and other tissues of certain animals (28) is attributable to discrepancies in the results obtained by different methods. A cys-

tine content of 1.05 per cent has been reported for salmon protein (29).

There is accumulating evidence that legume seeds are generally a poor source of dietary methionine, and Lugg & Weller (30) have suggested that the seeds and whole seed proteins of any legume should be deemed of low methionine content unless experimental evidence exists to the contrary. The work which leads to and supports this suggestion has come chiefly from two independent regions in which entirely different methods have been used. Thus Almquist and collaborators (31) and others (32, 33, 34) in the United States have tested the nutritive values of these materials for rats and chicks, whereas chemical methods of investigation have been used in Australia (35, 30, 36). The cyst(e)ine content varies widely (35, 30, 36). Incidentally, it has been found (37) that in ether-extracted pollens from various sources there is probably inadequate methionine (or cystine) for normal growth of rats.

New estimations of the cystine and cysteine contents of a number of keratins have been reported (38, 39). Methionine is perhaps a constant, if minor, constituent of keratins. About 1.0 per cent has been reported (40) in human hair. This is to be compared with estimates of 0.4 per cent (41) and 0.5 per cent (42) in wool, and 0.02 per cent in steer horn (43).

Recent claims have been made (44) for the occurrence of a thioiminazole derivative (possibly thiolhistidine) in serum proteins, or at least in serum peptides, which represents some 12 per cent of the organic sulfur. It has been suggested that djenkolic acid may be present in proteins, and that as it would yield cysteine and formaldehyde during acid hydrolysis, humin could result from the reaction between the formaldehyde and tryptophane (45).

*Biological occurrence of certain compounds.*—A convenient means of isolating crystalline glutathione from germinating peas, has been described (46).

Adenochrome, the red glandular pigment of the branchial hearts of the octopus, *Paroctopus bimaculatus*, has an elementary composition similar to that of urochrome, but all of its 5.67 per cent of sulfur is in the oxidized form (47).

Unidentified sulfur-containing substances, some of which are volatile in steam, are among the substances responsible for the odor and taste of the potato (48). Whereas many halophyte plants produce volatile organic sulfur-containing compounds, only one hydrophyte,

*Nasturtium officinale*, was found to do so (49). Milk has been found to contain up to 0.24 mg. of sulfur per litre in the form of heat-volatile sulfide (50).

Bufothionine was found to be commonly present among the bufo-toxins in the venoms of a number of South American toads (51).

*Structure and properties.*—In the course of a study of sulfhemoglobin formed *in vivo* Nijveld (52) has confirmed Michel's (53) observations that the substance contains, per atom of iron, one atom of sulfur more than does hemoglobin. Its divalent iron atom is readily oxidized to the trivalent condition by ferricyanide. The assumption (53), that sulfhemoglobin is formed when hydrogen sulfide and peroxide react with hemoglobin, is considered false (52).

It has been found (54) that the constitution of the 12-phosphotungstate of cystine, precipitated from hydrochloric acid solution, may be expressed as  $\text{RSSR}(\text{H}_3\text{PO}_4, 12\text{WO}_3)6\text{H}_2\text{O}$ .

Although in a general way the anticoagulant power of heparin increases with the sulfur content, this does not uniquely determine the activity (55). Jorpes (55) believes that the heparins from different species consist of one or more chondroitin—or mucoitin—sulfuric acids, but perhaps the most direct refutation of the view that "heparin" is a single substance has been provided by the fractionation of sodium salts (56). Attention may be drawn to recent work (57) on the constitution of chondroitin sulfuric acid (or sulfate) from cartilage. Among the more active synthetic compounds of the heparin type are chondroitin sulfate polysulfate, cellulose-hydrogen glycolate sulfate, and cellulose  $\beta$ -hydroxyethane sulfonate polysulfate (58).

Redox potentials of some sulphydryl-disulfide systems, cysteine, glutathione, thioglycolic acid, monothioethylene glycol, thiolhistidine, and other sulphydryl compounds, have been calculated from the results of electrometric titration with iodine or permanganate (59). The  $E_0$  values found are not in good agreement with those obtained by other workers using similar methods (60, 61). Discrepancies may be connected with the intrusion of the "anomalous" redox potential, concerning the nature of which (62) there is much disagreement.

Crystals of *dl*-methionine were found (63) to be monoclinic hemimorphic hemihedral, with axis ratios  $a:b:c = 2.09:1:3.43$ , and  $\beta = 102^\circ 7'$ . *l*-Cystine and *l*-methionine are among the  $\alpha$ -amino acids of which the rotary dispersions have been studied recently (64).

*Methods of estimation, techniques.*—Vassel's (65) method for the estimation of cystine and cysteine was found to be unsatisfactory with

hydrolysates of keratins which had been treated with certain detergents, and was modified (66) to make it more reliable. Tests of the recovery of cysteine under the conditions of acid hydrolysis of proteins, have shown that when protein is present the loss may be serious (67). Losses, particularly those encountered when carbohydrate is present, have been discussed in an earlier review (68). Pile & Routh (67) treat with reserve estimates of the cysteine content of protein preparations based upon acid hydrolysis. However, such estimations are being made with increasing frequency (e.g., 16, 39).

Studies of the sulfur distribution in plant tissues have been made (69), the fractions being: "labile-," sulfate-, soluble organic-, and insoluble organic-sulfur. In spite of the experience of Clarke & Inouye (70), Thomas & Hendricks (69) believe that close control of the alkaline decomposition, which yields "labile"-sulfur, permits a reasonably reliable estimate of any cystine present.

Of the microorganisms which will not grow unless certain amino acids are present, *Lactobacillus arabinosus* seems to be particularly easily adapted to the estimation of certain amino acids including cystine. Titratable lactic acid is produced in proportion to the amount of any essential amino acid that is added to the medium previously lacking that amino acid (71, 72, 73).

Like other thioethers, methionine reacts with iodine to form a periodide. By changing the pH the bound iodine can be liberated for titration. This procedure forms the basis of an interesting new method for estimating methionine (74). The originator of this method appears, however, to have wrongly interpreted Bailey's (75) findings to mean that methionine is not held by acid "humins."

A saturated solution of anhydrous cupric sulfate in concentrated surfuric acid is virtually colorless, and gives a yellow coloration with methionine (76). It is claimed to be an improvement upon the reagent of Kolb & Toennies (77), who used cupric chloride in hydrochloric acid, and suitable for detecting methionine in leucine preparations.

Considerable methionine has been found in the washed and reprecipitated cuprous cysteine mercaptide deposited from hair hydrolysate (40), and doubt has been thrown upon the validity of the method of calculating the cystine content from the organic-sulfur content of such precipitates.

A "partition" chromatographic method has been used for estimating the methionine in protein hydrolysates (41). The use of charcoal poisoned with cyanide, has been proposed (78) for the

chromatographic separation of methionine, cystine, and certain other amino acids from tyrosine, tryptophane and phenylalanine.

The rates of liberation of cystine or cysteine from various proteins during hydrolysis with solutions of different acids, have been investigated (79).

An improved Grote (80) reagent has been described (81) for the colorimetric estimation of thiourea in tissue fluids. Thiocyanates interfere by contributing a similar blue coloration. It is stated, however, (82) that even with the original Grote reagent the interference is negligible with comparable amounts of thiocyanate.

In continuation of the study of the use of hydrogen peroxide in formic acid solution (83) to destroy methionine in diets, Toennies & Homiller (84) have examined the performic acid content of the mixtures and the oxygen consumption of amino acids. Per molecule, *dl*-methionine consumed about two atoms of oxygen, *l*-cystine about five, and *l*-tryptophane about three, in one or two hours. Sulfuric acid can be used in place of formic acid when destroying the methionine and tryptophane in casein hydrolysates by oxidation with hydrogen peroxide (85).

*Reactions involving sulfhydryl groups and disulfide links.*—The oxidation in solution of reduced glutathione to the disulfide form by oxygen, which is known to occur readily in the presence of copper salt at pH 8.3, takes place also in the pH range 9 to 11, but in the pH range 11 to 13 oxidation proceeds further, apparently with the formation of the sulfinic acid of glutathione (86, 87).

It has been found (88) that the sulfhydryl groups of bisulfite-treated wool (89) can be methylated by methyl iodide or bromide, the S-cysteine sulfonate groups also present being unaffected by the methylating agent. The authors describe the isolation of S-methylcysteine from hydrolysates of the methylated material, by "partition" chromatography of the N-acetylated amino acids.

Jones & Mecham (39, 90), studying the dispersion of keratins, stress the view that the splitting of  $-SS-$  links and of secondary links in keratin are processes which can take place independently, dispersion resulting when both have occurred. They were successful in dispersing at neutrality keratins treated with bisulfite, thioglycolate, or monothioglycol in solutions of urea guanidine, acetamide, and other protein denaturants.

It has been contended (91) that the cystine links of keratin fibres are first hydrolysed by aqueous solutions of oxidizing agents and



then oxidized to sulfinic and sulfonic acids. Partial immunity to the attack of the oxidizing agents is conferred upon the fibre by first boiling it with dilute formaldehyde solution at pH 6 (92). Treatment with benzoquinone solution (93) restores the strength of bisulfite-treated keratin fibres. Mizell & Harris (94) have given support to the view (95) that alkali cleavage of  $-SS-$  links in wool involves rupture of one  $-CS-$  bond giving a dehydroalanine and a  $-CH_2-S-SH$  residue. These residues are believed to recombine after the latter has lost a sulfur atom, with formation of lanthionine. The hypothesis is in conflict with the views of Schöberl (96), who has shown, however, that a dehydroalanine residue may take part in the reactions. X-ray examination of alkali-treated wool indicates that the  $\alpha$ -keratin structure is retained and is thus independent of the actual cystine content (97).

Acylating agents like ketene, carbon suboxide, and phenyl isocyanate react at pH 5 to 6 more rapidly with the sulfhydryl than with the amino and phenolic groups of egg albumin (98). The thioethers so formed may be hydrolysed by alkali with almost complete restoration of the sulfhydryl groups.

The sulfhydryl groups liberated when tobacco mosaic virus (which normally appears not to contain them) was denatured with urea or guanidine hydrochloride, were found (99), particularly with the latter denaturant, to account for almost all the sulfur present. The liberation of sulfhydryl groups by heat denaturation of ovalbumin could be inhibited by the presence of *d*-mannitol or the sugars, *d*-glucose, *d*-fructose, *d*-mannose, and *l*-arabinose (100). Acid and alkali denaturation of ovalbumin decreased the sulfur content by about 7 per cent, but acid denaturation of edestin raised the sulfur content slightly, the protein losing some organic matter (101). Sulfhydryl group liberation and other aspects of protein denaturation have been discussed in a recent important review (102).

The frequently close connection between enzyme activity and sulfhydryl groups, present in the enzymes themselves or belonging to substances associated with the substrates, has long been recognized. It is now customary in describing and characterizing an enzyme, to refer to the effects (inhibition, activation) which sulfhydryl compounds exert upon its activity. Sometimes the effects of sulfhydryl-group destroyers (oxidizing agents, salts of mercaptide-forming metals, etc.) are reported. From the voluminous relevant literature, reference may be made to an extensive study by Barron & Singer (103) of the effects

of organic arsenicals, chloromercuribenzoate, and iodoacetamide (as sulfhydryl-group destroyers) upon some thirty enzymes. The hypothesis that activation of papain consists in reduction of disulfide links, is considered by Scott & Sandstrom (104) to be less satisfactory than their own activator-enzyme surface compound hypothesis, which they hold to be supported by the different activating effects of ethyl, propyl, butyl, amyl, heptyl, and benzyl mercaptans and other sulfhydryl compounds.

Mercaptide formation probably accounts for the observations that cystine and cysteine can diminish the toxicities of therapeutic compounds containing arsenic, bismuth, or gold (105). Mercuric acetate, however, retains high toxicity.

*Tribochemical and other investigations.*—In recent contributions (38, 106, 107, 108) to the tribochemistry of proteins, the sulfur and cystine contents of the modified proteins obtained by prolonged dry-grinding have been examined. Grinding greatly diminished the cystine content of keratins (38). The water-soluble protein obtained by grinding heat-coagulated ovalbumin had nearly twice the sulfur content of the unground material (106), but the water-soluble and water-insoluble fractions of ground casein showed greatly diminished sulfur contents (107).

Cathepsin from normal rat liver hydrolysed only the glycine peptide bond of glutathione (109), whereas cathepsin from rat hepatoma 31 (109) and extracts of ground rat kidney (110) hydrolysed both peptide bonds. Cystine was not liberated in peptic digests of various proteins but was rapidly freed during tryptic digestion (111).

In presence of the Raney nickel catalyst, hydrogen converts benzoyl-*dl*-methionine mainly into *dl*- $\alpha$ -(benzoylamino)-butyric acid, and benzoyl-*l*(—)-cystine mainly into benzoyl-*l*(+)-alanine (m.p. 144–45° C.), thereby confirming the view that *l*(—)-cystine and *l*(+)-alanine have the same absolute configuration (7).

#### METABOLIC ASPECTS

*Nutritional requirements.*—Rose and collaborators (112) have shown that the ten amino acids (including methionine) now considered indispensable for maintenance and satisfactory growth of experimental animals, are also indispensable in human nutrition. Again, experiments in which nitrogen-poor vegetables, and casein hydrolysates freed sometimes from methionine and sometimes from cystine, were used in the diets, have shown (113) that methionine is neces-

sary to maintain a nitrogen balance with human subjects. The experiments failed to prove, however, whether cystine is or is not essential.

The claim that dietary cystine is needed for regeneration of plasma protein in dogs (114) has been withdrawn (115), but the stimulatory effect of administered cystine upon the regeneration, even at expense of body tissue, has been affirmed. Incidentally, the rapid incorporation (compared with that of homocystine or methionine) of cystine, containing radioactive sulfur, into the plasma proteins of hypoproteinemic dogs (116) is in harmony with this. Protein containing radioactive sulfur in amino acid residues can be prepared from the plasma by dialysis.

The mouse, like the rat, can synthesize cystine from dietary methionine (117) and can utilize both *l*- and *d*-methionine for growth purposes (118). A severe metabolic disturbance, with slow growth, high mortality, and liver damage, has been encountered when rats are fed diets believed to be deficient in cystine (119). Addition of 0.2 per cent of *l*-cystine to the diet restored normal growth and mortality. Similarly, the failure of mice maintained on a low cystine diet for twenty-two months to have regular estrus cycles, could be prevented by adding 0.5 per cent of cystine to the diet (120).

Cystine supplementation reduced the amount of dietary casein required for optimal growth of rats under hot and cold conditions (121). Also, added *l*-cystine and *dl*-methionine had a marked protein sparing action for dogs maintained on low protein diets (122). With *dl*-methionine supplementation, in particular, the increase in the urinary organic sulfur fraction was large.

The suggestion (123), that alkaline conditions in the alimentary canal might convert some of the cystine in the dietary protein into lanthionine, has been followed by the announcement (124) that *meso*-lanthionine will not support the growth of rats receiving a cystine-deficient diet.

It has been shown (125) that choline-depleted chicks can utilize homocystine in place of cystine but not in place of methionine, that the chick can utilize *dl*-methionine for growth equally with the *l*-enantiomorph, and that S-methyl-cysteine can neither replace cystine in the diet nor assist in the utilization of homocystine. Thus, as earlier work with rats too has disclosed, the methyl group of S-methyl-cysteine lacks biological lability.

Disease conditions in the chick (the "cartilage factor" and the so-

called "vitamin B<sub>4</sub>" deficiency diseases) which are associated with inadequate intake of cystine, arginine, and glycine, or, perhaps, with the inability to utilize fully these substances when supplied in dietary protein, have been investigated (126, 127).

Both cystine and methionine are among the essential nutrilites for *Lactobacillus arabinosus* (71, 128). After repeated subculture of *L. casei*, this organism too is found to need methionine (129) as well as cystine (130), among other amino acids, for maximum growth. Cystine has been described as an essential nutrilitite for the growth of *Proteus morganii* (131), methionine being unable to replace it with full effectiveness. Glutathione is an essential growth factor for certain repeatedly subcultured strains of *Neisseria gonorrhoeae* (132), and incidentally, for mosquito larvae (133).

Among mutants of the mold *Neurospora crassa* induced by x-rays and ultraviolet radiation (134, 135), the strains which cannot synthesize methionine, leucine, and arginine, have been found to be capable (136) of utilizing racemic mixtures of the *d*- and *l*-forms of the amino acids as effectively as the physiologically normal forms. The  $\alpha$ -keto analogues of methionine and leucine can serve in place of the amino acids. These facts suggested that the mold might be able to convert the *d*- into the *l*-forms by oxidative deamination and re-synthesis, and it was indeed found to contain a *d*-amino acid oxidase which can rapidly effect the oxidative deamination of *d*-methionine.

*Labile methyl groups, lipotropic activity.*—The importance of the concept of the biologically labile methyl group as a dietary essential, playing rôles in nitrogen, sulfur, fat, and carbohydrate metabolism, has been emphasized by du Vigneaud (137). In man the S-methyl group of dietary methionine is used in the synthesis of choline and creatinine (138). In the rabbit it has been shown to be involved also in the synthesis of creatine and anserine (139). This was established by feeding deuteriomethionine and finding deuterium in the urinary creatinine and in the choline, creatine, and anserine of the tissues. Handler & Bernheim (140) have found that *d*(+)-methionine is only about half as useful as *l*(-)-methionine for creatine synthesis *in vitro* with rat-liver slices. The sulfoxide and sulfone of *dl*-methionine were ineffective, but the methyl sulfonium chloride and the  $\alpha$ -keto analogue of methionine were as active as methionine itself. The transfer of choline methyl groups for the biological synthesis of methionine, long presumed to occur from indirect evidence, has been confirmed directly by the finding of deuteriomethionine in the proteins of rats fed

homocystine and deuteriocholine (141). Following upon the demonstration that methionine is not an effective substitute for dietary choline in prevention of the slipped tendon syndrome (perosis) in chicks (142), it has been found that arsenocholine will prevent perosis but is almost ineffective as a methylating agent for homocysteine (143). The ability of homocystine sometimes to support the growth of rats in the absence of dietary methionine or choline (144, 145) may depend partly upon a slow release of methyl groups from other sources in the body.

Interesting transmethyations involving methionine have been reported for etiolated wheat germs (146). Creatine and betaine syntheses, when glycocyamine and glycine respectively were provided, were increased many fold by addition of methionine, and an obligatory oxidation (by oxygen) of the methionine sulfur was involved, a considerable fraction of it appearing as sulfate. In sharp contrast is the fact that oxidation of the methionine sulfur does not appear to occur in transmethylation *in vitro* with liver slices (140).

It has been suggested that the net lipotropic action of a protein in a diet is the balance between the opposing influence of the cystine and methionine in it (147). This suggestion has been affirmed by Treadwell, Groothuis & Eckstein (148) against opposition from different quarters (149, 150). It has been questioned again by Channon, Mills & Platt (151) who conclude that the lipotropic factors in proteins include cystine, methionine, possibly tyrosine, and one or more substances belonging to the butanol-soluble fraction of protein hydrolytic products. Recent studies (152), however, suggest that these divergent conclusions may be associated with the fact that the lipotropic action of dietary methionine is markedly affected by the abundance or lack of other essential amino acids. When these are plentiful, the lipotropic action of methionine is at a maximum and, contrary to previous understanding (148), possibly identical for free methionine and methionine in proteins.

Young rats maintained on a fatty-liver diet accumulated no additional liver fat when sodium sulfide (unlike cystine) was administered intraperitoneally. Dimethyl sulfide, dimethyl disulfide, S-methylisothiouraea, and methyl xanthogenate, like methionine, effected a decrease in liver-fat content, whereas methionine sulfone did not. Also, no decrease was effected when trimethyl sulfonium chloride was administered orally (153). Methionine sulfoxide, however, does show lipotropic activity (154). It is of interest to compare these results with the findings of related studies (e.g., 140, 142). The biological

lability of methyl groups (with which the lipotropic action of substances like methionine is believed to be associated), seems to depend considerably upon the nature of the transmethylation to be effected, but a satisfactory interpretation of the facts has not yet been provided. Whereas methionine may replace choline in prevention of the hemorrhagic kidney conditions of weanling rats maintained on a diet deficient in labile methyl groups, caffeine is much less effective (155).

*Urinary excretion.*—When adrenaline is administered to rabbits it is not conjugated with glucuronic acid for excretion, but the increase in organic sulfates excreted (156) suggests that there is conjugation with sulfuric acid.

The formation *in vivo* of N-acetylated, S-substituted cysteines, the mercapturic acids, continues to be investigated. Zbarsky & Young (157) have demonstrated the conversion of phenyl-*l*-cysteine into *l*-phenylmercapturic acid in rats. They have shown that benzene (158) takes its place with naphthalene (159) and anthracene (160) in giving rise to the corresponding phenylmercapturic acid, that *p*-fluorophenylmercapturic acid arises from the administration of *p*-fluorophenyl-*l*-cysteine (12), and that fluorobenzene takes its place with the other halogenated benzenes in giving rise to the corresponding *p*-fluorophenylmercapturic acid (161).

Stekol (162) has confirmed the assumption of White & Jackson (163) that the inhibition of growth of rats which are fed bromobenzene, is due to depletion of available cysteine by mercapturic acid formation.

Phenyl- and benzyl- *d*-cysteine were excreted by rats mainly as the corresponding N-acetyl-*l*-amino-acids (164), but N-acetyl benzyl-*d*-cysteine itself was excreted without inversion.

In dogs with lobar pneumonia caused by Type I pneumococcus, urinary excretion of inorganic sulfate and of organic sulfur compounds increased sharply, whilst that of ethereal sulfate and of thiosulfate was scarcely affected (165). The male dogs normally excreted about ten times as much thiosulfate as the females did.

As the result of further work with cystinuric dogs, Hess & Sullivan (166) have advanced the generalization that when the sulfur atom, the nitrogen atom, or both, of ingested cysteine is bound to some other group there is little, if any, formation of extra cystine.

In studies of the metabolism of carbon disulfide in man and other animals, it has been found (167) that most of the retained vapor is converted into inorganic sulfate and organic sulfur compounds, which are excreted in the urine.

*Other metabolic investigations.*—Discussing possible metabolic paths for the formation of taurine from cystine and cysteic acid in the liver, Medes & Floyd (168) have stated that thiopyruvic acid, arising from transamination reactions, appears not to be involved in sulfate formation. Cysteine has been added (169) to the sulfur compounds (sulfate, deaminated methionine) which are produced when methionine is incubated with liver slices. Hydrogen sulfide under similar conditions yields sulfur, polythionates, and sulfate (170). It has been claimed that cysteine desulfurase and homocysteine desulfurase are not identical (171), and that cystine and homocysteine must first be reduced to the sulphydryl condition before the respective enzymes will liberate hydrogen sulfide from them (172). In the metabolism of the autotrophic bacterium, *Thiobacillus thiooxidans*, the energy yielding process (oxidation of sulfur to sulfuric acid) may be separated in time from the assimilation of carbon dioxide. The energy, meanwhile, is stored in adenosinetriphosphate. This interesting discovery has been discussed fully in a recent review (173).

In a recent report (174) attention has been drawn to the ability of microorganisms of the *Salmonella* group to reduce tetrathionate to thiosulfate. The production of hydrogen sulfide by certain Gram-negative organisms in the presence of cystine, methionine, cysteic acid, and other sulfur compounds, has been studied (175, 176). Verona (177) has reviewed the biological production of hydrogen sulfide.

Although sunflower plants grown on sulfur-deficient nutrient solution were smaller and produced smaller seeds than normal plants, the percentage of sulfur in them and the percentage germination were not affected (178). The presumption is that any composition differences were probably small. On the other hand, when selenates were applied to alfalfa plants growing on soils believed to be deficient in sulfur, the ratio of nitrogen to organic sulfur in the plants fell even lower than it did with gypsum manuring (179). It would be of great interest to ascertain whether any fraction of the decrements in this ratio was due to change in composition (increase in cyst(e)ine or methionine content) of the protein present in the plants.

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COMMONWEALTH COUNCIL FOR SCIENTIFIC AND INDUSTRIAL RESEARCH  
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# THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS

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During the past year considerable new knowledge has been added to that previously available concerning the function of phosphate compounds in metabolism. The well-known relationship between oxidation and phosphorylation has been further consolidated and extended, and some completely new reactions involving phosphate transfer have been discovered, such as the cleavage of thio-ether linkages by adenosine-triphosphate, and the ability of the latter to phosphorylate pyridoxal.

Inasmuch as other chapters in this volume are devoted to nucleic acids and phospholipoids, only brief mention will be given in this chapter to studies dealing with these compounds. It is hardly necessary to add that European literature during the year is covered only incompletely due to the difficulty of obtaining European journals. For the same reason it has been necessary to include European publications which are one or two years old. My thanks are due to Professor J. Runnström, Wennergren Institute of Experimental Biology, Stockholm, Sweden, for valuable information regarding research in Sweden during the past few years.

## DETERMINATION OF PHOSPHATE

A modification of the usual method for determination of phosphate has been described by Borei (1). Norberg (2) describes an ultramicro determination of total phosphorus using a photoelectric microscope described by Caspersson (3). Lowry & Bessey (4) have devised another ultramicro phosphorus determination using the Beckman spectrophotometer and narrow cells (1 or 2 mm. wide and 10 mm. deep).

The method for phosphoglycerol determination has been found to lack specificity (5). Haas (6) has described a spectrophotometric micro method for the determination of glucose-6-phosphate using dichlorophenolindophenol as hydrogen acceptor and the specific dehydrogenase as catalyst. The method is also useful for the study of dehydrogenase.

## PHOSPHATASES

*Determination.*—The King-Armstrong (7) method for the determination of phosphatase has been modified by Binkley, Shank & Hoagland (8). Phosphotyrosine is used as substrate and the liberated tyrosine is determined colorimetrically. By this method phosphatase can be determined in 1 ml. of plasma. Determination of phosphatase in very small quantities of blood has been made possible by the ingenious method of Lowry & Bessey (4) in which a nitrophenyl phosphate reagent is used. Three c.mm. of plasma at pH 10 are incubated with 50 c.mm. of the reagent, and after the addition of sodium hydroxide the intensity of the color (400 m $\mu$ .) produced by the liberation of the free sodium salt of nitrophenol is measured directly in a photoelectric colorimeter. The method is simple and direct, and particularly useful when large numbers of determinations are required.

Menten *et al.* (9) have modified the histochemical phosphatase test. They used a mono-aryl phosphate which is hydrolyzed by phosphatase. The liberated aryl group reacts with a diazotized amine, forming a highly colored insoluble dye. Barium- $\beta$ -naphthol phosphate was used as the phosphoric ester and the diazotized  $\alpha$ -naphthol amine as the coupling substance.

Histochemical techniques have been used by a number of investigators to study phosphatases in normal and pathological tissues. Wilmer (10) using a histochemical technique found that the renal tubules in the cortex of aglomerular kidneys do not contain alkaline phosphatase, whereas the connective tissue contains considerable amounts of the enzyme. This finding is of interest in connection with the mechanism of sugar reabsorption proposed by Lundsgaard (11) which involves a phosphorylation-dephosphorylation cycle. If this theory is correct, it is only logical that kidneys in which no filtration takes place, and consequently no reabsorption, are devoid of phosphatase. The occurrence of an acid phosphatase in these kidneys still remains a possibility. Wilmer (10) also found a decrease in phosphatase in the tubular cells when the tubular function was destroyed, as for instance in hydro-nephrosis or spontaneous interstitial nephritis. Wachstein (12) studied renal phosphatases in hemorrhagic kidney due to choline deficiency. He found a marked decrease in alkaline phosphatase in the damaged atrophic tubules and some increase in phosphatase in the vessels and in the glomeruli. Alkaline phosphatase was also found in ovarian follicles and corpora lutea (13). Bodian & Mellors (14) found a marked

increase in acid phosphatase in the region of the cytoplasm of nerve cells in which chromatolysis had been produced by axon section. Adrenalectomy caused a decrease in kidney and liver phosphatase (15). The phosphatase content returned to normal following injection of adrenal cortex extract. Inclusion bodies of vaccinia, herpes simplex, fowl pox, etc. were also investigated by histochemical techniques and found to contain no phosphatase (16).

Changes in serum phosphatase under various conditions have been studied by a large number of investigators. Drill *et al.* (17) found that sodium cyanide in concentrations of 0.0001 to 0.1 molar has only slight inhibitory effect on serum phosphatase activity of normal dogs. However, when the serum phosphatase values were increased as a result of liver damage, the addition of sodium cyanide inhibited the extra phosphatase activity markedly. Gould (18) found that fat feeding causes an increase in serum phosphatase.

Herbert (19) found that prostate phosphatase can be distinguished from other acid serum phosphatases by its great lability. Thus one hour incubation of serum at 37° at pH 7.4 inactivates the prostate phosphatases, whereas the other acid phosphatases are not affected. Addition of 0.4 vol. of ethanol to prostate phosphatase in buffers from pH 4.8 to 7.4 renders it completely inactive after half hour incubation at room temperature, whereas the other acid phosphatases remain unaltered.

Delory & King (20) have studied the kinetics of phosphatase action at different pH's and with different substrates. They found that the rate of hydrolysis of phosphate esters having low ionization constants is higher than that of esters having high constants. Moreover, the pH optimum is more alkaline for the former than for the latter. These observations are consistent with the hypothesis of Martland & Robison (21) that the enzyme is a weak base whose undissociated molecule combines with a substrate to form a compound which breaks down into the products of hydrolysis. Alkaline phosphatase is apparently only active if amino groups as well as phenol groups are intact (18). Its behavior towards ketene treatment is similar to that of the lactogenic hormone.

Phosphatase from rat sarcoma has a pH optimum of 5.6 in the absence of magnesium or manganese. In the presence of those two metals the pH optimum is around 4.8 (22).

The more specific phosphatases will be dealt with in another chapter.



## FORMATION OF PHOSPHATE ANHYDRIDES

Mann (23) has conducted some interesting studies on the phosphorus metabolism of the mold *Aspergillus niger*. A specific metaphosphatase was isolated from extracts of the mold. Inorganic pyrophosphate and polymetaphosphates were isolated as ammonium and barium salts. The metaphosphates are readily converted into inorganic pyrophosphate during the isolation procedure. The metaphosphate could be separated from the inorganic pyrophosphate by chemical procedures as well as by enzymatic techniques. Thus pyrophosphatase splits only inorganic pyrophosphate, whereas the metaphosphatase from mold hydrolyzes metaphosphate as well as pyrophosphate. The metaphosphate isolated acted as a precipitating agent for proteins. Inorganic pyrophosphate has been isolated from yeast (24), and recently Cori & Ochoa (25) have isolated considerable amounts of inorganic pyrophosphate from liver extracts which oxidized dicarboxylic acids. It is not definitely established yet whether the pyrophosphate, the polymetaphosphate or perhaps an organic complex represent the compound originally present in the cell.

PHOSPHATE COMPOUNDS AND ENZYMES INVOLVED IN  
CARBOHYDRATE METABOLISM

*Formation of phosphohexoses.*—Phosphorylase has been studied by Sumner, Somers & Sisler (26). They found that the nature of the products synthesized from Cori ester by plant phosphorylase depends upon the kind and amount of polysaccharide added to prime the reaction. Thus a small quantity of achroodextrin will lead to the production of a substance giving a blue color with iodine. A larger quantity of achroodextrin will cause a product to be formed giving a red color with iodine, while addition of a very large amount of the dextrin will cause the production of a substance which gives no color at all with iodine. In each case the quantity of inorganic phosphate liberated is practically the same. The authors interpret their findings as follows: the enzyme adds anhydro-*D*-glucose molecules to whatever foundation it finds present. If a few dextrin molecules are present it forms a chain sufficiently long to give a blue color with iodine. If many dextrin molecules are present, phosphorylase forms many polysaccharide chains of intermediate length and the product resembles erythro-dextrin. In this connection the experiments of Hidy & Day

(27) are of interest. They find that the priming effect of polysaccharides is markedly increased by partial hydrolysis. The staining reaction with iodine disappears completely about the time the polysaccharide begins to decline in activating power. All these observations are in agreement with the formulation proposed by Cori *et al.* (28) as a result of their experiments with crystalline muscle phosphorylase incubated with varying amounts of glycogen. Cori *et al.* formulated the polysaccharide formation catalyzed by phosphorylase as follows: glucose-1-phosphate plus terminal glucose units  $\rightleftharpoons$  maltosidic chain units plus inorganic phosphate. The terminal glucose units are the end groups of the highly branched glycogen molecule which serves as the primer of the polysaccharide formation.

Sumner & Somers (29) have described a simplified method for the preparation of glucose-1-phosphate employing purified potato phosphorylase.

Doudoroff, Hassid & Barker (30) described the synthesis of two new sugars which appear to be analogs of sucrose. A phosphorylase prepared from *Pseudomonas saccharophila* catalyzes the reversible splitting of sucrose into Cori ester. If *l*-sorbitose or *d*-ketosylsucrose is incubated with the enzyme in the presence of Cori ester, a reaction similar to that observed in the presence of fructose and Cori ester takes place, i.e., inorganic phosphate is liberated and the amount of sugar decreases. The synthetic compound appears to be the disaccharide glucoside-sorbitide.

Shapiro & Wertheimer (31) investigated phosphorylase activity in various animal tissues. They found a highly active phosphorylase in subcutaneous tissue. Phosphorylase was not found in the muscle of rats of less than ten days of age. In fourteen-day-old rats the enzyme is already active. No decrease in the glycogen phosphorylase of muscle could be demonstrated in adrenalectomized or in thyroidectomized rats. The inhibitory effect of glucose on muscle phosphorylase was diminished by adenylic acid. No glucose inhibition was found with potato phosphorylase.

*Transphosphorylation of hexoses.*—The transphosphorylation of glucose to hexose monophosphate and of the latter to hexose diphosphate by adenosinetriphosphate has been given much attention during recent years. Youngburg (32) studied aerobic phosphorylation of sugars in kidney cortex extract. He found that whereas hexoses were readily phosphorylated, no phosphorylation of pentoses took place.

Klein (33), studying the metabolism of brain tissue, observed that the oxidation of fructose is accompanied by a phosphorylation. Huszak (34) found that the white and gray matter of the brain show a different carbohydrate metabolism. The white matter used preferentially glycogen as metabolite, whereas free glucose was not utilized. The main metabolite in the gray matter was glucose, which was phosphorylated by adenosinetriphosphate and subsequently oxidized. Phosphopyruvic acid is also able to phosphorylate glucose, but only through the adenylic acid system. Lindberg (35), working in Runnström's Institute, studied the carbohydrate metabolism of sea-urchin eggs during fertilization. He found that the dehydrogenases from ground sea-urchin eggs are strongly stimulated by the addition of hexose phosphates, phosphoribose, and phosphogluconic acid. Lindberg furthermore described a phosphoric ester, occurring in sea-urchin eggs, which has an activating effect upon the carbohydrate metabolism. This phosphoric ester was also found in and isolated from beef brain. The phosphoric ester was crystallized both as a brucine and an acridine salt, and the molecular weight was reported to be about 150. The substance, which is acid stable, behaves in many ways like glycerophosphate. In experimenting with egg pulp, it was found that the ester had a strongly enhancing effect on respiration. Furthermore, it caused a temporary accumulation of pentoses and of an unidentified acid. The amount of acid formed was of the same order of magnitude as that of the carbohydrates broken down. The author compared the phenomenon with that which occurs after fertilization. In both processes formation of acids takes place presumably by oxidative decarboxylation of hexose. Greville & Lehmann (36) studied phosphate and carbohydrate metabolism in extract of human muscle. They observed the well-known transphosphorylations and dephosphorylations.

The enzymes catalyzing the phosphorylation of hexoses to hexose mono- or diphosphates are of considerable interest for an understanding of the regulation of carbohydrate metabolism. Until recently the phosphorylation of glucose to hexose monophosphate (catalyzed by the enzyme hexokinase) had never been clearly demonstrated in muscle extracts. The phosphorylation of hexose monophosphate to diphosphate by adenosinetriphosphate was demonstrated in muscle extracts several years ago by Ostern (37) and his co-workers. Now, Colowick & Price (38) have succeeded in demonstrating hexokinase in extracts from rat muscle. They furthermore report that the transphosphorylation of glucose and hexose monophosphate requires the presence of

reduced cozymase, oxidized cozymase being without activating effect. The coenzyme of the transphosphatase seems to be destroyed rapidly by an enzyme in the muscle extract which has not yet been identified. However, it is known that animal tissues contain a specific nucleosidase which splits off the pyridine base in pyridine nucleotides (39). The finding that the reduced cozymase is the activator of these transphosphorylations may be of importance for the understanding of the so-called Pasteur effect, i.e., the suppression of fermentation by oxygen. In this connection some recent experiments of Engelhardt & Sakov (40) are of interest. They found that the addition of phenol, phenol oxidase, and the cytochrome system completely inactivates the transphosphorylation of hexose monophosphate. Kubowitz (41) has shown that the phenol oxidase system can reoxidize reduced cozymase.

The nature of the coenzyme of the hexose monophosphate fermentation (42) has not been further clarified.

Hotchkiss has continued his investigation of the effect of gramicidin on bacterial metabolism (43). He found that gramicidin increases the oxygen uptake of intact bacteria, provided glucose is the substrate, and that the uptake of phosphate is completely inhibited. In kidney extract, Hotchkiss (44) was able to show that the aerobic phosphorylation of glucose is also completely inhibited in the presence of small amounts of gramicidin (30 to 40  $\mu\text{g. per ml.}$ ).

Meyerhof & Beck (45) have purified the phosphotriose isomerase by ammonium sulfate fractionation and adsorption on cupric oxide. The preparation obtained was free of phosphohexose isomerase and of aldolase. The activity of the preparation was high but the stability low.

*Coupling between oxidation-reduction and uptake or liberation of phosphate.*—The enzymatic formation of 1,3-diphosphoglyceric acid (phosphoglycerylphosphate) from phosphotriose discovered by Warburg & Christian (46) and Negelein & Brömel (47) has been reviewed in previous volumes. Bücher, continuing these studies, has purified and crystallized the enzyme catalyzing the equilibrium between diphosphoglyceric acid and the adenylic acid system (48). The enzyme was precipitated as a nucleoprotein from acidified alcoholic solution and subsequently crystallized in alkaline ammonium sulfate (0.6 saturated) containing inorganic pyrophosphate. This enzyme is the most active fermentation enzyme thus far isolated. An amount as small as 0.01 mg. per ml. can be detected readily in the optical test at 334 m $\mu$ . (the test for reduced cozymase). The equilibrium catalyzed by the enzyme can be expressed as follows: 1,3-diphosphoglyceric acid plus

adenosinediphosphate  $\rightleftharpoons$  3-phosphoglycerate plus adenosinetriphosphate.

Lipmann has continued his studies of the formation and the properties of acetyl phosphate and has been able to throw light on many interesting problems concerning bacterial metabolism. Acetyl phosphate was synthesized by Lipmann & Tuttle (49) according to a greatly simplified method in which monosilver dihydrogen phosphate reacts with acetyl chloride yielding monoacetyl phosphate. Acetyl phosphate is readily hydrolyzed both in the acid and in the alkaline range. At pH 5 the compound showed maximum stability. The addition of substances which combine with phosphate greatly increases the hydrolysis of acetyl phosphate. Thus molybdate increases the hydrolysis of acetyl phosphate in acid solution, whereas calcium ions which precipitate phosphate at alkaline reaction correspondingly increase the hydrolysis of the compound in the alkaline range.

The formation of acetyl phosphate by oxidation of pyruvate in the presence of dry bacteria (*Bacillus acidificans longissimus*) has also been described by Lipmann (50). Acetyl phosphate was isolated as a silver salt and identified as disilver monoacetyl phosphate. In analogy with the findings of Bücher (48), it was found that acetyl phosphate is also able to transfer its phosphate group to the adenylic acid system.

Hitherto the enzymatic formation of acetyl phosphate had been demonstrated only in the special system just mentioned. However, during the last year acetyl phosphate formation has been observed in preparations from other bacteria. Koepsell, Johnson & Meek (51) have succeeded in demonstrating the formation of acetyl phosphate in the oxidation of pyruvate by a dry preparation of *Clostridium butylicum*. They found that in the absence of glucose, inorganic phosphate is taken up and appears as labile phosphate. After fractionation with silver they found that the purified labile phosphate fraction contained both acetic and butyric acid, this fact indicating the formation of both acetyl and butyryl phosphates. If butyric acid was incubated with acetyl phosphate in the presence of the enzyme extract, considerable amounts of butyric acid were found in the silver precipitate. This was interpreted as indicating the presence of butyryl phosphate since absorption of free butyric acid by the silver precipitate had been excluded. They suggested the following reaction: acetyl phosphate plus butyrate  $\rightarrow$  acetate plus butyryl phosphate.

Acetyl phosphate has also been shown to play a role in the phosphoroclastic splitting of pyruvate into acetate and formate catalyzed

by an enzyme from *Escherichia coli*. Utter & Werkman (52) found that the splitting of pyruvate proceeds according to the equation: pyruvate plus phosphate  $\rightleftharpoons$  acetyl phosphate plus formate. Utter, Werkman & Lipmann (53) have been able to show that this phosphoroclastic splitting is reversible. An enzyme preparation from *E. coli* was incubated with formic acid containing an excess of  $C_{13}$ , and pyruvic acid containing ordinary carbon. After one hour the  $C_{13}$  concentration in the formate had decreased considerably and was accounted for in the carboxylic group of the pyruvate. The carbon dioxide did not contain any excess  $C_{13}$ , indicating the absence of Woods' equilibrium enzyme (54). Although the equilibrium of the phosphoroclastic reaction is far toward the formation of acetyl phosphate, it has nevertheless been possible to demonstrate chemically the formation of small amounts of pyruvate by incubating acetyl phosphate and formate with the enzyme preparation (55). The equilibrium constant is roughly  $10^{-2}$  for the reversed phosphoroclastic reaction.

Acetyl phosphate may also play a role in animal tissue. Lipmann (56) has most recently described an enzyme occurring in skeletal muscle which rapidly and specifically dephosphorylates acetyl phosphate. The stability of the enzyme toward acid as well as its high specificity are features not ordinarily found among phosphatases. Its presence in tissue might very well interfere seriously with any demonstration of acetyl phosphate formation in animal tissue. The occurrence of such an enzyme in animal tissue, on the other hand, may also suggest that this compound actually is an intermediate in the carbohydrate metabolism of higher animals.

The role of acetyl phosphate in the formation of acetylcholine is not known. However, it has been found that adenosinetriphosphate under anaerobic conditions greatly stimulates the formation of acetylcholine in brain extracts (57, 58).

Ochoa (59) studied  $\alpha$ -ketoglutarate dehydrogenase from cell free suspensions of washed heart muscle. The  $\alpha$ -ketoglutarate was oxidized only one step, i.e., to succinate and carbon dioxide, provided that the succinic dehydrogenase was inhibited by malonate. In the presence of glucose, three mols of phosphate were transferred to the sugar (forming hexose diphosphate) for each mol ketoglutarate oxidized to succinate and carbon dioxide. Synthetic succinyl phosphate did not give rise to any phosphorylation of sugar but was rapidly dephosphorylated. Inorganic phosphate, magnesium ions, and muscle adenylic acid, or adenylypyrophosphate, were required for the activity of  $\alpha$ -ketoglutarate

dehydrogenase. Adenosinetriphosphate was five times as efficient an activator as adenylic acid. This difference was attributed to destruction of adenylic acid by deaminase action.

Long (60) studied the oxidation of  $\alpha$ -ketobutyrate on minced pigeon brain. He likewise found inorganic phosphate to be an essential component in the oxidation of pyruvate as well as of  $\alpha$ -ketobutyrate. Adenine nucleotides markedly increase the oxidation of pyruvate, provided inorganic phosphate is present. The oxygen-pyruvate ratio is 1:2 for the part of the pyruvate oxidation catalyzed by adenine nucleotides.

Leloir & Muñoz (61) have studied the oxidation of butyric acid in liver extract. They found that the oxidation of this fatty acid is stimulated by the presence of a number of dicarboxylic acids. All the active dicarboxylic acids when added alone to liver extract are readily oxidized and give rise to the formation of phospho-enol pyruvic acid. No phosphopyruvate is formed in the absence of adenylic acid, cytochrome-*c*, or inorganic phosphate. Malonate inhibits phosphopyruvate formation from succinate, fumarate, or citrate. Phosphopyruvate can replace dicarboxylic acids in increasing the rate of butyrate oxidation, provided that carbon dioxide is present, indicating that phosphopyruvate may be carboxylated.

Lehninger (62) has studied fatty acid oxidation in homogenized liver preparations. He found that the oxidation of saturated fatty acids having four to eight carbon atoms by homogenized rat liver requires the presence of adenosinetri- or adenosinediphosphate. Adenylic acid is inactive. This finding is in agreement with the observations of Lang (63) and of Shapiro & Wertheimer (64) who demonstrated that palmitic acid dehydrogenase from liver extract requires the presence of adenylypyrophosphate.

Lardy, Hansen & Phillips (65) found a phosphate uptake in sperm cells which is coupled to oxidations other than those of carbohydrate metabolism. The utilization of phospholipids was suggested as a possibility.

*Enolase*.—The work of Warburg & Christian (66) on crystalline enolase was reviewed last year (67). It will be recalled that the amount of magnesium present in purified enolase was analyzed and found to be one gram atom magnesium per 52,000 grams enolase. Bücher (68), working in Warburg's laboratory, has recently determined the molecular weight of crystalline enolase and found it to be 62,000, which means that one molecule of enolase when fully activated

by magnesium contains one atom of the metal. Bücher likewise found that the crystalline mercury salt of enolase contains one atom of mercury per molecule of enolase. The molecular weight of enolase was determined by a specially constructed apparatus applying the Tyndall effect as a measure of molecular size. Edestin, the molecular weight of which was determined by diffusion and sedimentation as well as by the Tyndall method, served as a standard. Bücher found that the molecular weight of the enolase decreased after dialysis at pH 5 and the activity disappeared; if salt were added the molecular weight increased to the original value and activity was fully restored.

### NUCLEOTIDES AND NUCLEIC ACIDS

*Analysis of nucleotides and nucleic acid in tissues.*—Analyses of tissues like muscle, liver, and kidneys have been made both by ordinary chemical methods, by enzymatic methods, and by optical methods. Caspersson & Thorell (69) using the photoelectric quartz microscope studied the ultraviolet absorption at various wavelengths of muscle fibers from *Drosophila funebris* which possesses large segments, making it possible to investigate the isotropic and anisotropic sections separately. He found that the ratio between the absorption at 260 m $\mu$ . to that at 280 m $\mu$ . was much higher in the isotropic than in the anisotropic part, indicating that the adenine nucleotides (which according to Parnas (70) constitute more than 90 per cent of the muscle purines) may be confined exclusively to the isotropic part of the resting muscle. There is reason to believe, as pointed out by Bernal (71), that myosin is present in both parts. Hoagland, Lavin & Shank (72) have reached the same conclusions as Caspersson, using a direct technique by which one part of the muscle fiber is photographed in polarized light and the rest of the fiber is simultaneously photographed in ultraviolet light (73). With this method, it was shown in human muscle fibers that the dark isotropic sections continue into the ultraviolet field as dark absorbing bands. It is hardly necessary to add that without the knowledge gathered by chemical analysis of muscle tissue, we would not be in a position to interpret the optical analyses.

A number of investigators have studied acid labile phosphorus in various tissues under various conditions. Wagtendonk (74) found a particularly marked lowering of the labile phosphate in liver and kidney of guinea pigs on a diet deficient in the so-called "antistiffness" factor. Whether the decrease in labile phosphate in this case is spe-



cific or nonspecific, it is difficult to decide. It is known that starvation of animals gives rise to the same phenomenon (75, 76). However, Wagtendonk's observations are of interest because the changes are much more marked than those observed in starved animals. There is reason to believe that the decrease in the labile phosphate is mainly due to a decrease of adenylypyrophosphate since the "adenylic acid fraction" was correspondingly increased. However, it would be of interest to know whether it is adenylic acid or inosinic acid which constitutes the main component of the so-called "adenylic acid fraction."

Rapoport *et al.* (77) found that red cells obtained during phenylhydrazine reticulocytosis showed a substantial increase in the concentration of adenylypyrophosphate as related to the hemoglobin.

Kabat (78) found an increase of acid labile phosphate in the brain of animals infected with poliomyelitis. Whether this labile phosphate can be identified with adenylypyrophosphate remains to be seen.

More precise information about the content of nucleotides in small tissue samples can be obtained by using enzymatic methods, provided the enzymes employed are sufficiently purified. Thus Schmidt & Engel (79) in 1933 initiated new methods for purine analyses in tissue samples by using purified deaminases and measuring the ammonia liberated from various purines. It has recently been possible to measure minute amounts of purine derivatives using Schmidt's enzymatic technique combined with ultraviolet spectroscopy. Thus a highly sensitive and specific method for the determination of muscle adenylic acid was developed by observing the change in the ultraviolet spectrum (the decrease in absorption at 265 m $\mu$ ) which takes place when the nucleotide is deaminated by adenylic acid deaminase (80). If the deaminase has been freed from impurities of myokinase, no decrease of absorption takes place when adenosinetri- or diphosphate is added. However, if a few micrograms of a specific adenylypyrophosphate from potato are added to the system, adenylic acid is formed and subsequently deaminated, thus causing a fall in the absorption. By this method less than 0.5 mg. of muscle tissue can be analyzed for adenylic acid and adenylypyrophosphate (81). A similar sensitive method for hypoxanthine compounds has been developed (81) using the rise in absorption at 290 m $\mu$  which takes place when hypoxanthine is oxidized to uric acid by xanthine oxidase. Inosine requires the presence of nucleosidase, and inosinic acid requires both nucleosidase and phosphatase in addition to xanthine oxidase before any rise in absorption will take place. This combination of optical and enzymatic methods

might be a valuable tool for studying changes in the composition of nucleotides or nucleic acids, as well as their enzymes in animal tissue under pathological conditions.

Anfinson (82) studied the distribution of diphosphopyridine nucleotide (DPN) in retina using the Cartesian diver technique; triose-phosphate dehydrogenase was used as catalyst. The higher concentrations of DPN (4  $\mu$ g. per mg. fat free solid) were found in the two synaptic regions. The rods and the outer nuclear layer contained less and the nerve fibers were very low in DPN.

Davidson & Waymouth (83) studied the content of nucleotides and nucleic acids in various tissues by means of ordinary chemical methods. The concentration of nucleotides seems to be lower in tumor tissue than in the corresponding normal tissues.

*Enzymatic reactions involving adenosinetriphosphate.*—Adenosinetriphosphate can participate in reversible transphosphorylations and in irreversible transphosphorylations, and it can undergo simple hydrolysis. The first type of reaction includes the phosphorylation of amidines (creatine, arginine) and of carboxylic groups (phosphoglyceric acid, acetic acid), and the phosphorylation of adenosinediphosphate (phosphate dismutation). The irreversible transphosphorylations include the phosphorylation of hydroxy groups such as the 1- or 6-hydroxy groups of hexoses and that of pyridoxal. The phosphorylation of hexoses has already been discussed. The phosphorylation of pyridoxal is a very recent observation (84) and of great interest because the phosphorylated product is active as a coenzyme of the enzyme which brings about decarboxylation of tyrosine. A completely new type of transphosphorylation was discovered by Binkley (85), who found that the terminal group of adenosinetriphosphates splits the thio-ether linkage of cystathionine with the formation of cysteine and phosphohomoserine. The reaction is a strictly stoichiometric one, with one mol of phosphate being transferred per mol of cysteine liberated.

*Adenylpyrophosphatases.*—A highly active adenylpyrophosphatase was isolated from potatoes (86). The enzyme splits both labile phosphate groups from adenosinetriphosphate. It is activated by calcium ions. Inosinetriphosphate is hydrolyzed to inosinic acid. The rate is somewhat slower than for adenylpyrophosphate. The enzyme is readily adsorbed on myosin, a phenomenon of interest in the discussion of the possible identity of myosin with muscle adenosinetriphosphatase (ATP-tase) (87). Myosin might be identical with ATP-tase

but the possibility that the muscle ATP-tase is adsorbed on myosin has certainly not been excluded. The muscle deaminase has also been found in myosin even after three reprecipitations (88).

A large number of investigators have continued the study of the myosin adenylypyrophosphatases. Ziff & Moore (89) studied myosine ATP-tase by means of electrophoresis and ultracentrifugation. Myosin was found to consist electrophoretically of one component to which 90 per cent of the triphosphatase activity is associated.

The effect of oxidation and reduction on myosin ATP-tase has been studied extensively. Singer & Barron (90) found that mercaptide-forming compounds as well as mild oxidizing agents inhibit ATP-tase. This inhibition was attributed to an oxidation of sulphydryl groups since the inhibited enzyme could be reactivated by adding reduced glutathione. They found a close parallelism between the number of sulphydryl groups attacked by mercuric *p*-chlorobenzoate and the degree of inhibition of enzyme activity, and interpret these findings as evidence in favor of Engelhardt's hypothesis that muscle ATP-tase is identical with myosin. Ziff (91) found that stored myosin loses its ATP-tase activity but can be partly reactivated by cystine or glutathione, which also reactivates oxidized myosin. Mehl (92) observed a greater decrease in ATP-tase activity in stored rat muscle myosin when the activity was estimated at a pH of 9 than when determined at pH 6 or 7. He likewise found that oxidation and reduction have a much greater effect on the activity measured in the alkaline range than when measured in the acid range. Binkley, Ward & Hoagland (93) studied myosin from persons afflicted with hereditary muscle dystrophy and found that the preparation contains an active ATP-tase. It was found that traces of copper completely inhibited the enzyme activity. The effect of copper was nullified by the addition of cyanide. Cyanide also increased the activity of fresh myosin preparations as well as preparations inactivated by oxidation with hydrogen peroxide. They devised a method of purification in which the myosin was precipitated with copper and redissolved in cyanide buffer. In this way they succeeded in obtaining ATP-tase preparations of more constant activity.

As might be expected, myokinase added to myosin ATP-tase results in the dephosphorylation of adenosinediphosphate (ADP) (86). This is merely due to the enzymatic conversion of ADP into adenylic acid and ATP, which is then subsequently hydrolyzed by ATP-tase.

Dainty *et al.* (94) studied the particle shape of myosin by means

of anomalous viscosity and flow birefringence. When a myosin solution was incubated with a small amount of ATP its birefringence was decreased about half, and the relative viscosity was slightly decreased. The full effect of ATP was obtained at a 0.004 molar concentration. Although other substances can cause a decrease in flow birefringence, to do so they must be present at a much higher concentration than this. The changes of physical and chemical properties of myosin brought about by ATP-tase are spontaneous and reversible, and seem to be connected with the enzymatic action of the protein as an ATP-tase. Effects similar to those of ATP have been obtained so far only with inosinetriphosphate, whereas inorganic triphosphate, although hydrolyzed by myosin phosphatase, has no effects on the physico-chemical properties of myosin.

Important contributions to our knowledge in this field have also been made from the Institute of Medical Chemistry in Szged by Szent-Györgyi and his group (95). They observed a marked difference in the physico-chemical as well as the enzymatic properties of myosin, depending on the method of extraction. The myosin obtained by extracting skeletal muscle ten minutes with potassium chloride shows a low viscosity; this preparation is called myosin "A" by the Hungarian group. If the muscle, on the other hand, is extracted for several hours with alkaline potassium chloride, a myosin preparation is obtained which is highly viscous and which readily forms fibers when injected into distilled water. The second type of myosin is called myosin "B" (96). If a myosin B fiber is placed in a freshly prepared water extract of muscle it contracts and becomes opaque. A myosin A fiber shows no change under such circumstances. Three components are necessary for the effect on the myosin B fiber: potassium, magnesium, and adenosinetriphosphate (ATP). If a contracted myosin B fiber is subsequently suspended in 0.2 M potassium chloride (containing magnesium ions) and ATP is added, the fiber relaxes. However, if the relaxed fiber is suspended in 0.1 M potassium chloride (containing magnesium ions) addition of ATP now produces contraction. Thus ATP addition can give rise to either contraction or relaxation, depending upon the potassium chloride concentration. In order to bring a contracted fiber into a state of relaxation in the absence of ATP, potassium chloride concentrations as high as 0.6 M are required, and it is necessary to adjust to a quite alkaline pH range.

Straub (97) has isolated a protein called "actin" from muscle which is soluble in alkalis. An actin solution remains liquid in the absence

of salts. Upon addition of salt, the viscosity as well as the birefringence is greatly increased. Actin is able to combine with myosin, forming more or less viscous complexes, depending on the viscosity of the original "actin." For a given actin preparation the maximal viscosity is reached by mixing one part actin to three parts of myosin, a ratio which according to the authors is very nearly the same as that found in skeletal muscle. If ATP is added to a viscous solution of an actin-myosin complex it causes a marked decrease in viscosity, approaching the viscosity found for myosin A. Szent-Györgyi concludes from this observation that ATP separates myosin B into actin and myosin A. After ATP has been hydrolyzed by phosphatase action, the viscosity is found to increase again, indicating that after the disappearance of ATP, actin and myosin A are again able to form a complex (myosin B). One mol ATP is able to effect a decrease in viscosity of 100,000 grams of myosin, which indicates that if the molecular weight of myosin is around 100,000, one mol of ATP reacts with one mol myosin (98). Szent-Györgyi emphasizes that the ATP reacts with the myosin component and not with the actin component. This is further indicated by the fact that ATP is also able to decrease the viscosity of free myosin (myosin A) in salt solution.

Szent-Györgyi interprets the difference in myosin obtained by various methods of extraction as follows. Extraction of fresh muscle, containing a large amount of ATP, with saline yields myosin A, leaving the actin in the insoluble residue. If, on the other hand, the muscle is extracted with alkaline potassium chloride overnight, the ATP is hydrolyzed, and a myosin-actin complex, myosin B, is obtained. Suspensions of myosin A or B in potassium chloride solutions of a strength between 0.1 and 0.2 M are flocculated by the addition of ATP. Suspensions of myosin in potassium chloride solutions stronger than 0.2 M go into solution upon addition of ATP, an effect which is otherwise obtained only by raising the potassium chloride concentration to 0.6 M and making the reaction alkaline. These observations are similar to those just described for the myosin fiber.

The effect of ATP on isolated muscle fibers has also been studied (99). It was found that minute amounts of ATP applied directly to the isolated muscle fiber cause a rapid contraction. Intra-arterial injection of ATP likewise gives rise to muscle contractions accompanied by electrical activity (100). The curarized muscle is just as sensitive to ATP as the non-curarized. ADP has the same effect as ATP, whereas adenylic acid, though just as effective in the non-curarized

muscle, is much less effective in the curarized. Inorganic phosphate is inactive, whereas inorganic triphosphate and pyrophosphate release contractions. In smooth muscle, only ATP is active.

*Pharmacological effect.*—Green & Stoner (101) report that the toxic effect of ATP on rats is potentiated by magnesium salts. The studies of Bollmann & Flock (102) are of interest in connection with the problem whether ATP plays a major role in tourniquet shock and traumatic shock. They found that ATP is almost completely hydrolyzed in muscles deprived of their blood supply. If the occlusion lasts more than three hours, little if any rephosphorylation takes place after the blood supply has been re-established. The decomposition products are relatively nontoxic and, presumably, consist mainly of inosinic acid. Tourniquet shock is, therefore, not caused by release of adenylic acid derivatives into the blood stream. Moreover, it is unlikely that adenylic acid compounds play a major role in traumatic shock, even though it has been found by means of the spectroscopic deaminase method just described (80) that the adenosine derivative concentration of the blood coming from a traumatized extremity is increased (103). That this influx of adenosine compounds is insufficient to exert a depressor effect is shown by the failure of injections of adenosine deaminase plus phosphatase to effect the low blood pressure accompanying traumatic shock (103). And yet, this enzyme combination exerts a marked antagonistic effect on the fall in arterial blood pressure caused by infusion of ATP. Release of adenylic acid or its derivatives into the blood stream seems, therefore, to be at the most a secondary factor in the traumatic shock.

*Prosthetic groups.*—Phosphorylated compounds have been identified as prosthetic groups in two important enzymes. Ratner *et al.* (104) found that the flavine adenine dinucleotide is the prosthetic group of glycine oxidase. Gunsalus *et al.* (84) found that phosphorylated pyridoxal can activate the decarboxylation of tyrosine. They were able to obtain an active coenzyme, both by chemical phosphorylation as well as by enzymatic phosphorylation, using ATP as a phosphate donor.

The experiments of Westenbrink & Veldman (105) indicate that phosphothiamine synthesized in the yeast cell is not all bound to carboxylase, although it is present in a form in which it is attacked much more slowly by yeast phosphatase than is free phosphothiamine. Thus the phosphothiamine content of yeast may be increased 1700 per cent without increase in carboxylase activity. One must, however, bear in

mind that phosphothiamine seems to be the prosthetic group of a number of dehydrogenases as well as carboxylases.

*Nucleic acids.*—Davidson & Waymouth (106) studied a factor in pancreatin which increased nucleoprotein phosphorus of fibroblasts *in vitro*. The active material seems to be a mixture of polypeptide and nucleotide derivatives. Gulland *et al.* (107) reviewed critically previous claims concerning the structure of nucleic acids in the dividing cell. Claude (108) found nucleic acid associated with the formed elements of the cell. Woodward (109) observed ribonuclease in the plague bacillus. Bain & Rusch (110) described a manometric determination of ribonuclease.

*Desoxyribonucleic acid.*—Avery, MacLeod & McCarty (111) isolated in a highly purified form a factor from type III pneumococci which is able to transform the unencapsulated R variant of *Pneumococcus* type II into the fully encapsulated cells of type III. The active factor was shown to be a highly polymerized specific desoxyribonucleic acid which is destroyed by phosphatase and by minute amounts of purified desoxyribonuclease. The wide significance of this work will be discussed elsewhere.

#### PHOSPHOLIPIDS AND THEIR CONSTITUENTS

Baer & McArthur (112) have synthesized phosphorylcholine by phosphorylation of choline halide with diphenylphosphoryl chloride in pyridine, with subsequent isolation of the diphenylphosphorylcholine as the chloroaurate. The latter compound is decomposed with metallic silver, yielding the free diphenylphosphorylcholine which in turn may be readily catalytically hydrogenated to the free phosphorylcholine.

Riley (113) studied the metabolism of phosphorylcholine. The betaine is readily dephosphorylated *in vivo* and the inorganic phosphate is excreted in the urine. Phosphorylcholine exerts an inhibition of the turnover of phospholipid in the liver. The inhibition appears to be limited to the noncholine phosphatide fraction. Phosphorylcholine, as a unit, is probably not utilized in the synthesis of phospholipids. Wagner-Jauregg & Lennartz (114) have synthesized dicholesterol pyrophosphate.

Elliott & Lebet (115) have studied oxidation of phosphatides in brain extract. Ascorbic acid and iron salts or iron protein complexes greatly stimulate the oxygen intake.

STUDIES OF THE METABOLISM OF PHOSPHATE  
COMPOUNDS IN VIVO

*Studies of carbohydrate metabolism in vivo by means of radioactive phosphate.*—A number of publications dealing with the study of carbohydrate metabolism and with the rejuvenation *in vivo* of phosphate compounds such as adenosine polyphosphates and phosphocreatine have appeared this year. It will be recalled that Sacks in 1940 (116) expressed the belief that, contrary to what had been found *in vitro*, the formation of lactic acid in the working muscle *in vivo* is independent of phosphorylations. The main evidence brought forward against the occurrence of a phosphate cycle in the working muscle was the finding that phosphocreatine and pyrophosphate from the muscle of animals injected with radioactive phosphate contained the same concentration of  $P_{32}$  whether the muscle was working or resting, that is, whether much or little lactic acid was being produced. The isotope concentrations of the two fractions were much lower than that found in the inorganic phosphate. Bollmann & Flock (117) obtained essentially the same results and drew the same conclusions. It was, however, soon realized that the conditions under which these experiments were conducted did not permit valid conclusions about the rate of phosphate turnover in the phosphate compounds of the working muscle. The main difficulty encountered in these studies has always been, and still is, to obtain reliable figures for the isotopic concentration of the inorganic phosphate in the muscle fiber. The reason for this difficulty must first of all be attributed to the very slow penetration of phosphate into the muscle fiber (Hevesy, 118). As a consequence of this, the isotopic concentration of the extracellular inorganic phosphate is manifoldly higher than that of the inorganic phosphate inside the muscle fiber. The very low isotope concentration of pyrophosphate and phosphocreatine as compared to inorganic phosphate, observed by Sacks, and Bollmann & Flock must, therefore, be ascribed to a contamination of the cellular inorganic phosphate with the highly radioactive extracellular phosphate. In a later paper Sacks & Alt-schuler (119) have taken the extracellular phosphate into account but, nevertheless, still maintain that there is an essential difference in metabolism between the resting and the working muscle with respect to the phosphate cycle.

It seems justifiable to raise the question whether there actually is any conflict between *in vitro* and *in vivo* studies in this special case.



So far, we do not know of any experimental data from *in vivo* studies of phosphate metabolism which cannot be accounted for by the so-called Embden-Meyerhof scheme.

One way of studying the rate of rejuvenation of phosphate compounds in muscle is to remove the extracellular phosphate by perfusing the muscle with saline. If, under these circumstances, the  $P_{32}$  concentrations of phosphocreatine and adenylypyrophosphate are compared with that of the true intracellular inorganic phosphate, one finds a very rapid rejuvenation of the phosphate compounds of the muscle (120). Thus only twenty minutes after an intravenous injection of  $P_{32}$  the phosphocreatine phosphorus has an isotope concentration about 60 per cent that of the inorganic phosphate; the same results are obtained for adenylypyrophosphate. In other words, the phosphorus turnover of this labile phosphate compound is already so high in resting muscle that with present techniques it would be quite difficult to discover any substantial increase in the turnover during or after muscular work. This technical failure does not, however, justify us in claiming that a further increase of phosphorus turnover does not actually take place during muscular contraction. The rate of rejuvenation of adenylypyrophosphate phosphorus and of phosphocreatine phosphorus in resting rabbit muscle amounts to 20 to 30  $\mu\text{g}$ . phosphorus per minute per g. of muscle, and there is every reason to expect that the rate will be manifoldly higher in working muscle. In the liver the rate of rejuvenation of adenylypyrophosphate phosphorus is about the same order of magnitude as that of resting muscle.

The rate of rejuvenation of the two labile phosphates in adenosine-triphosphate has been studied using hexokinase as an instrument to differentiate between the terminal and the second phosphate group (121). In the resting rabbit muscle the  $P_{32}$  concentration was always found to be the same in both of the labile phosphate groups even when investigated shortly after the injection of radioactive phosphate. However, Flock & Bollmann (122), using myosin ATP-tase as a tool to differentiate between the two labile groups of ATP, have found a distinctly higher  $P_{32}$  concentration in the terminal phosphate group as compared with that of the second group.

In the studies of Kalckar *et al.* (120) the relative isotope concentration of hexosemonophosphate phosphorus in resting muscle varied greatly but was usually found to be considerably lower than that of the pyrophosphate. However, in certain cases the isotope concentration of this ester (which was purified as the calcium salt and "washed" with

inert inorganic phosphate) was found to be as much as three times that of the pyrophosphate and more than twice that of the inorganic phosphate (120). The same observation has been made by Sacks (119), who has been able to throw further light on this finding (123, 124). The interpretation of Sacks' figures are, however, somewhat difficult because no values for the  $P_{32}$  concentrations of the inorganic phosphate were presented. Undoubtedly, the most important observation is that the high  $P_{32}$  concentrations of the "glucose monophosphate" phosphorus are never found in fed animals (in the post-absorptive phase) but only in fasted animals. The results might be interpreted as indicating that, before entering the cell, some form of hexose combines with extracellular phosphate, which is, of course, very rich in isotopic phosphate. The possibility suggested by Hotchkiss (43) that phosphate from the environment reacts with a polysaccharide complex in the cell wall of microorganisms forming a hexosephosphate might very well be considered in the case of muscle. Sacks discusses a similar hypothesis. The observations made by Myrbäck & Vasseur (125) that certain enzymes in the yeast cell seem to be located inside and certain other enzymes outside a barrier may also be of interest in this connection.

Kaplan & Greenberg (126, 127) have published a number of papers dealing with the determination of phosphoric esters in liver by barium and mercury fractionation, and by hydrolysis curves. The justification for identifying the "seven minutes acid-hydrolyzable phosphorus" in the fraction of water-insoluble barium salt with the labile phosphorus of pyrophosphate is open to criticism. The identification of these two fractions is justified when applied to acid filtrates from skeletal muscle, but the picture in the liver is much more complex. Inorganic pyrophosphate, for instance, has been found in liver (25), and other yet unknown acid labile phosphoric esters having insoluble barium salts may belong to this fraction. The authors report that insulin (in the presence of glucose) increases the amount, as well as the  $P_{32}$  content, of the labile groups of ATP. The changes observed are small and may be secondary or nonspecific. It is known that reduction of food intake causes a decrease of the acid-labile phosphorus in the liver (75), an observation actually confirmed by Kaplan & Greenberg (76). It is, therefore, conceivable that the effect of insulin may be the result of its known effect in increasing the food intake.

*Rejuvenation of phosphorus in nucleic acids.*—Brues *et al.* (128) investigated the turnover of phosphorus in the desoxyribonucleic acid

(nuclear nucleic acid) from liver and found that the rate of rejuvenation in non-growing liver is very slow (10 to 11 per cent rejuvenation after three days). As shown by Marshak (129) the rate of rejuvenation of ribonucleic acid (cytoplasmic nucleic acid) phosphorus is very rapid. Euler & Hevesy (130, 131, 132) studied the rate of rejuvenation of nucleic acid phosphorus in tumors. The radioactivity per mg. nucleic acid phosphorus was compared with that of the free inorganic phosphate. In two hours 2 to 3 per cent of the total nucleic acid phosphorus of a Jensen sarcoma had been rejuvenated. The phosphorus of desoxyribonucleic acid of growing sarcoma was also turned over at a considerable rate (1.5 per cent per hour). Irradiation with 1,000 International Roentgen units decreased the rate of rejuvenation of nucleic acid phosphorus to half or one-third of that of the untreated sarcoma. This effect of x-rays appears before the fall in the number of mitoses occurs.

*Phosphate turnover of phospholipids.*—Two independent groups have confirmed and extended previous reports (133) that choline deficiency causes a decrease in the synthesis of phospholipids in the liver and kidney of young rats in which damage of the two organs has been produced by the dietary regime. Patterson *et al.* (134) found the rate of rejuvenation of phospholipid phosphorus decreases in choline deficient rats. Boxer & Stetten (135) found correspondingly that the daily replacement of choline in phospholipids, which in normal rats amounts to 3.9 mg., is decreased to one-third in choline deficient rats. The effect consists in a retardation of the incorporation of new choline into the phosphatide without altering the quantity of choline present in the phosphatides.

The reader is referred to the chapter on lipid metabolism for further information about this interesting topic.

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# CARBOHYDRATE METABOLISM

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## ENZYMES AND ISOLATED SYSTEMS

*Phosphorolysis and synthesis of higher sugars.*—Doudoroff and his co-workers have described an enzyme which catalyzes the phosphorolysis of sucrose, yielding glucose-1-phosphate and fructose (1 to 4). The enzyme, obtained from *Pseudomonas saccharophila* Doudoroff, was prepared nearly free of invertase or phosphatase, appeared to require no coenzyme, and was inhibited by glucose. Only sucrose was phosphorylated by the enzyme, maltose, trehalose, raffinose, glycogen, or starch not being attacked. In the reverse reaction, glucose-1-phosphate was used specifically; the fructose could not be replaced by an aldose nor by fructose phosphate, but evidence of synthesis of sugars from glucose-1-phosphate and *l*-sorbose or *d*-ketoxyllose was obtained. When the enzyme was allowed to act on glucose-1-phosphate and fructose, the crystalline product isolated appeared by all tests to be identical with naturally occurring sucrose (5). Sucrose phosphorylase appears to be parallel in function to the phosphorylases of animal tissue and plants which act on glycogen and starch. The action of such an enzyme may explain the more rapid use of sucrose than of glucose or fructose by certain organisms; and since this reaction is easily reversible, it provides a mechanism for the synthesis of the compound sugar. The occurrence of phosphorylases for sucrose or other disaccharides in other plant and animal forms would be of considerable general significance.

The view that synthesis of polysaccharides by phosphorylase consists of the reaction of glucose-1-phosphate with the terminal units of the polysaccharide present has received further support in the observation that the synthesis is enhanced by dextrans (6) or by partly hydrolyzed fractions of amylose (7). According to Hestrin & Avineri-Shapiro (8), the synthesis of the fructo-polysaccharide levan from sucrose or raffinose by preparations from *Aerobacter levanicum* does not require inorganic phosphate. The authors consider that the intermediate in this case is not a phosphorylated hexose but is some de-



ivative of fructose having a relatively high energy content and a function analogous to that of a phosphate ester.

*Glycolytic system.*—Meyerhof & Beck (9) have prepared triose phosphate isomerase in fairly pure form, free of aldolase and of hexose phosphate isomerase. The enzyme was found in high concentration in rabbit muscle, constituting about 4 per cent of the protein, and it appeared to have a very high turnover number. Such concentration and activity could help to overcome the unfavorable position of the triose phosphate equilibrium, in which only 4 per cent of the triose exists in the form of glyceraldehyde phosphate. Because the enzyme transferring phosphate from the adenylic system to fructose-6-phosphate was quite susceptible to oxidizing agents and since certain oxidizing agents inhibited the fermentation of glucose but not that of hexose diphosphate, Engelhardt & Sakov (10) suggested this enzyme as the point in the glycolytic scheme at which the Pasteur effect is initiated. From a comparison of the rates in minced tissues of certain reactions of the glycolytic system with the rates of oxygen uptake and the extent of inhibition of respiration by sodium fluoride, Breusch & Peters (11) concluded that the phosphorylating system can be the main pathway of carbohydrate oxidation only in striated muscle. In other organs (brain, liver, kidney, spleen, pancreas, salivary gland, lung, placenta) sodium fluoride had little effect on respiration, or the rates of the reactions studied were slow. Since the minced tissues were incubated in different dilutions of different media in the several types of experiment, the comparisons of rates must be considered rough approximations only. It is also possible that substances other than carbohydrate were being oxidized by some of these tissue samples. Chesler & Himwich (12), studying the rate of glycolysis in various parts of the central nervous system in cats and dogs, found that the region having the highest rate appeared to advance rostrally as growth proceeded. The rate of glycolysis fell with growth (from one week to three months) in the cord and medulla, but increased with growth in the higher portions (cortex, thalamus). In rat brain slices the total energy which could be derived by oxidation of carbohydrate (in phosphate buffer) appeared to be about four times that which could come from anaerobic glycolysis (in bicarbonate buffer) (13). The efficiency of these processes may vary with the medium and other conditions, so this ratio would not necessarily indicate the relative amounts of energy actually made available by the two processes *in vivo*. In homogenized suspensions of cat brain, the principal steps in the metabo-

lism of fructose (aerobic and anaerobic) were found to be essentially the same as those occurring in glycolysis (14). The failure of brain *in vivo* to use fructose as readily as it does glucose was assumed to be due to slow entrance of fructose into the cells. Some of the principal reactions of the glycolytic cycle have been shown to occur in human tissues (15).

*Oxidation of carbohydrate.*—Ochoa (16) has studied the properties of an enzyme in cell-free suspensions of washed heart muscle which catalyzes the oxidative decarboxylation of  $\alpha$ -keto glutaric acid to succinic acid. Cytochrome-*c* was shown to be the physiological carrier between this system and oxygen, while 4-carbon dicarboxylic acids did not catalyze hydrogen transport. The system could phosphorylate glucose, although the further oxidation of succinate was inhibited by malonate; the ratio of phosphorus esterified to oxygen consumed was the same as in pyruvate oxidation, namely 1.6:1 observed, probably 3:1 if corrected for phosphatase activity. Inorganic phosphate, magnesium ions, and adenylic acid or adenosinetriphosphate were required by this enzyme. Evidently this step in the Krebs cycle could be one of those responsible for the generation of high energy phosphate bonds in the oxidation of pyruvate. It seems likely that but one such bond could be generated by the primary dehydrogenation step, the others being formed during the course of hydrogen transport. Ball (117) has discussed the thermodynamic relationships of the intermediates in the oxidation of carbohydrate and pointed out that the largest part of the energy of this oxidation must be realized in the steps following the initial dehydrogenation.

Lipman has succeeded in isolating the acetyl phosphate formed on the oxidation of pyruvic acid by a bacterial enzyme system (from *B. acidificans longissimus*) (17, 18). Another enzyme preparation (from *E. coli*) catalyzes the phosphoroclastic splitting of pyruvate, forming acetylphosphate and formic acid (19, 20). This reaction was shown to be reversible, whereas the decarboxylation of pyruvate by hydrolysis is essentially irreversible. In certain organisms, formic acid is in equilibrium with carbon dioxide, so that it would be possible for carbon dioxide to be incorporated into the pyruvic acid molecule by the action of this system. Since formic acid is energetically equivalent to a mixture of carbon dioxide and hydrogen, the reversibility of the phosphoroclastic reaction suggests that the oxidative decarboxylation of pyruvic acid to acetyl phosphate may also be reversible. Lipman (18) also demonstrated the enzymatic transfer of phosphoryl

groups from acetylphosphate to adenylic acid. The reaction was measurably reversible when adenosinetriphosphate acted as phosphate donor. Utter & Werkman too found the phosphoryl group of acetylphosphate transferable to glucose and to adenylic acid in extracts of *E. coli* (21). The generation of high energy acyl phosphate bonds by oxidative phosphorylation or by phosphorolysis of pyruvate is important in simple systems and has great theoretical interest; but whether direct oxidation of pyruvic acid to acetylphosphate or to acetic acid regularly occurs in animal tissues is not yet decided. Bloch & Rittenberg (22) studied the sources of acetic acid in animals by determining the amounts of deuterium found in the acetyl derivative of phenylaminobutyric acid when the latter compound was fed along with other substances containing deuterium. The demonstration that a large fraction of the alanine fed could form acetyl derivatives was considered evidence that the pyruvic acid formed from alanine was oxidized to acetic acid in the animal body. That acetic acid itself was the acetylating agent is uncertain; it may have been acetylphosphate, or it may have been some other compound formed in the cyclic oxidation of pyruvic acid.

In circumstances in which the oxidative phosphorylation of glucose readily occurred in rabbit kidney cortex, the pentoses *l*-arabinose, *d*-ribose, and *l*-xylose were not phosphorylated (23). An apparent catalytic effect of biotin on the oxidation of pyruvate and lactate by the livers of biotin deficient rats was noted by Summerson *et al.* (24). Breusch, using the Thunberg technique, detected in cat liver a new series of dehydrogenases acting on *d*-arabinose, glyceraldehyde, glycolaldehyde, *d*-erythrose, and *d*-sorbitol. The intermediate acids which would be expected to be formed were not further dehydrogenated under these conditions (25).

The enzymatic decarboxylation of oxaloacetate by cell-free preparations from *E. coli* was studied by Kalnitsky & Werkman (26). Magnesium ions were required by the enzyme, but cocarboxylase and phosphate were not. The reverse reaction, the condensation of carbon dioxide with pyruvate, was believed to occur, the oxaloacetate formed being measured by two different analytical techniques. The equilibrium condition of this reaction is far on the side of decarboxylation, and Evans, in his review of carbon dioxide fixation (118), calculated that the amounts of oxaloacetate reported by Kalnitsky & Werkman were about one thousand times larger than the maximum to be expected under their experimental conditions. According to Evans,

either pyruvate was not the true substrate for the carboxylation or most of the oxaloacetate must have come from other reactions. The possibility exists that this reaction is not a simple decarboxylation but one which can be reversed by coupling with an exergonic system. Since the enzyme preparation of Kalnitsky & Werkman was not highly purified and other reactions, such as the formation of acetylphosphate from pyruvate could occur (21), these authors suggested that energy for the synthetic reaction may have been furnished by such a process.

#### GENERAL INTERMEDIATE METABOLISM

The rate of incorporation of stably bound deuterium into carbohydrate and fat *in vivo* has been utilized by Stettin & Boxer to calculate the rates of turnover of liver and carcass glycogen and of liver fat in rats (27). When the animals were kept at constant weight on a high-carbohydrate, fat-free diet, about 69 per cent of the liver glycogen appeared to be replaced daily, as was 19 per cent of the carcass glycogen and 37 per cent of the liver fat. Since the apparent maximal content of deuterium in the glycogen was about half the theoretical amount, the authors concluded that formation of glycogen from glucose (which would not take up much isotope) and formation from smaller units had been about equal in rate. From the amounts of glycogen recovered and from its isotope content, it was calculated that under these conditions less than half a gram of glycogen had been newly synthesized per day. This figure is perhaps somewhat too low, since the animals were stunned rather than anesthetized at the time the samples were taken; but in any case the amount of glycogen synthesized would still be but a small fraction of the amount of carbohydrate which the animals consumed each day (about 14 gm. glucose monohydrate, equivalent to 11 gm. of glycogen). Evidently most of the carbohydrate fed must either have been oxidized directly or have been deposited (temporarily) as fat. From data quoted from another source, the authors calculate the daily replacement of fat, presumably by synthesis from carbohydrate, to have been about 2 gm. While the amount of carbohydrate required to make this amount of fat is not known, not less than 3 and probably nearly 5 gm. (if 2 of each 3 carbon atoms were available) would have been utilized in fat synthesis, as compared to the fraction of a gram laid down daily as glycogen. Considering the relative magnitudes of the storage facilities for fat and for carbohydrate in the well nourished animal, this is not truly a surprising finding, but its demonstration in quantitative terms emphasizes the

importance of fat formation as a pathway of carbohydrate metabolism.

In a second paper Boxer & Stetten determined the rate of incorporation of deuterium into the glycogen formed in the feeding of glucose and of lactate to fasted rats (28). Glycogen containing deuterium was formed at very much higher rates than in the well nourished animal in a steady state, this fact indicating that even after the feeding of glucose a high proportion of the new carbohydrate had been synthesized from fragments smaller than glucose. When lactate was fed, it appeared that at some time nearly all of the hydrogen atoms must have been exchangeable with those of water.

From their study of the metabolism of mannose, Bailey & Roe (29) concluded that except for its slow rate of absorption from the gut, it was well utilized. Doses of 2 to 5 gm. per kg. administered intravenously to rabbits were retained almost entirely, and liver glycogen was formed readily. The glucose and lactate content of the blood rose, but no fructose appeared. Galactose when fed to rats formed both glucose-1-phosphate and galactose-1-phosphate in the liver and the glucose of the blood increased; glucose-6-phosphate was believed to be the intermediate (30). Cellobiose fed to rats was absorbed at a slow rate, the rate of digestion possibly being the limiting factor; but the deposition of glycogen and anti-ketogenic and nitrogen sparing effects were similar to those of glucose (31). The  $\delta$ -lactone of gluconic acid was found by growth tests to serve as well as glucose as a source of energy in the diet of young rats when given in rather small amounts, rations sufficient for optimal growth not being given because of the laxative effects (32). The same may be true for lactose and  $\beta$ -lactose. When fed as the chief source of carbohydrate, these substances caused severe diarrhea and were inadequate for growth (33), although they seem to be well utilized when supplied as dietary supplements.

The frequent occurrence of abnormal glucose tolerance curves and reduced deposition of liver glycogen in certain strains of rats has been reported in past years. Sayers *et al.* (34) found that when rats of this type (Yale strain) were adrenodemedullated, their tolerance to glucose became normal. An hereditary hyperactivity of the adrenomedullary system in this strain of rats would explain adequately the previous findings, although some degree of participation of other factors, such as the adrenal cortex, cannot yet be excluded. Lewis *et al.* (35) presented data on the effects of excessive dietary sodium or potassium in rats on the glucose tolerance curves, respiratory quotient and liver glycogen deposition after glucose feeding, and on sensitivity to insulin.

The authors considered that in general high sodium diets tended to lower the rate of oxidation of fed carbohydrate and to increase its storage as glycogen. Since changes in the ionic environment might be expected to influence particularly the metabolism of the peripheral tissues, data on muscle glycogen formation and on the metabolism of the tissues in these conditions would be desirable for complete interpretation of the data. From determinations made by Hard *et al.* (36) on the fat and carbohydrate content of the livers of fetal and newborn guinea pigs and on the respiratory quotients of newborn animals, it would appear that these animals used carbohydrate as a primary fuel only during the first hours after birth. Thereafter, for some days, the large stores of fat previously laid down seemed to be used preferentially.

*The kidney in carbohydrate metabolism.*—Kidney slices incubated in serum formed glucose at a rate exceeding that shown by liver slices in the same medium (37). The suggestion has been made that the effects of nephrectomy in increasing the apparent glucose utilization rates of eviscerated animals may be due to the removal of the kidney as a source of new glucose (38, 39). A small renal arteriovenous difference in fermentable reducing substances was observed by Reinecke in eviscerated rats previously fasted twenty-four hours (39). Roberts & Samuels (40) were able to demonstrate a consistent renal arteriovenous difference averaging 18 mgm. per cent of glucose in eviscerated rats fasted two days, but they did not observe any arteriovenous differences in unfasted eviscerated rats, or in intact animals, either fasted or fed. Since no renal arteriovenous differences in amino nitrogen were found in any case, the authors suggested that substances other than amino acids must have been the source of carbohydrate. However, only very small differences in amino nitrogen content could have been expected (not more than 3 to 5 mgm. per cent) if only blood amino acids were used, and it is possible that tissue amino acids or polypeptides in blood or tissue may have served as the chief source. It is difficult to explain why no contribution of carbohydrate by the kidneys was observed in the intact or in the fed eviscerated rats. If the higher glucose requirements of nephrectomized-eviscerated animals are in fact due to the removal of a site of gluconeogenesis, then considerable quantities of carbohydrate must be furnished by the kidneys of fed as well as of fasted eviscerated rats (38, 41). The question may be raised whether the kidney normally releases glucose in intact animals. In the absence of the liver, gluconeogenesis might be stimulated

by an increase in the amino acid level of the blood. However, the increased glucose requirement after nephrectomy is evident within a short time after removal of the liver from the circulation, before the amino nitrogen content of the blood has risen noticeably (42). Therefore, unless some other explanation exists for the increase in glucose requirement, gluconeogenesis by the kidney must occur at normal as well as at high blood amino acid levels. It is possible that lactic acid may be a precursor of glucose in the kidney, especially if the concentration of lactic acid in the blood is high as it is in many eviscerate preparations.

Since fructose will prolong the life of eviscerated animals and kidney tissue may convert fructose to glucose, Reinecke (43) attempted to determine whether the absence of the kidneys would alter the metabolism of fructose in eviscerated rats. The author recorded small decreases in the rate of disappearance of fructose from the body and in the response of the blood sugar and lactate levels to the injection of fructose. Unfortunately, the charts in which the data are presented were reduced to such a scale that it is impossible to judge the significance of the changes observed.

*Effects of the nutritive state.*—It has long been known that after fasting, undernutrition, or diets low in carbohydrate, administered carbohydrate appears to be less rapidly dealt with than normally. The metabolism of both the liver and the peripheral tissues appears to be altered by these procedures (see below), but to what extent these changes are due to a relative lack of or delay in insulin secretion and what part may be the result of adaptation to the previous conditions by tissues other than the pancreas is not known. In normal men not taking other food, the ingestion of 25 to 35 gm. of glucose was required daily to maintain normal glucose tolerance curves (44). Insulin administration, presumably simulating fasting by maintaining a low blood sugar level for some time, may cause a reduction in the subsequent tolerance for glucose in men (45) and in chicks (46). Fasted rats were found to be considerably less sensitive to insulin if they had previously been on low carbohydrate regimens, probably because such animals have much larger amounts of glycogen in their livers than do rats which have been fed high carbohydrate diets before being fasted (47). Roberts *et al.* (48) found that when rats which had been maintained on high fat diets were eviscerated, the blood sugar fell more slowly than it did in rats previously fed high carbohydrate diets. Since the metabolic rates and the rates of increase in blood nonprotein nitro-

gen were the same in both types of preparations, the authors concluded that a more rapid rate of fat metabolism persisted in the absence of the viscera in rats which had previously been using mostly fat. This difference may possibly have been the result of the persistence of large amounts of insulin in the tissues of the carbohydrate-fed animals as compared to the fat-fed rats; but the experiment to decide this point—evisceration of previously depancreatized animals maintained on the different diets with constant amounts of insulin—has not been done. A similar but less marked difference in the rate of fall of the blood sugar after evisceration was observed by Reinecke & Roberts (41) between rats fasted two or four days and rats not fasted before operation. In rats which were also nephrectomized, no such difference was discerned; but the survival time of the animals in both series was perhaps too short to allow proper assessment of the contribution of the kidneys. However, as noted above, Roberts & Samuels (40) found that the kidneys of fasted eviscerated rats released sugar into the blood, but those of fed rats did not. The authors concluded that fasting had increased the rate of gluconeogenesis in the kidneys, as well as in the liver, and that this fact could account for part of the apparent difference in glucose utilization rates between fasted and fed eviscerated rats.

Recently, Kaplan & Greenberg (49) compared the rates of incorporation of radioactive phosphorus by the liver in rats which had been maintained on high fat, high carbohydrate, or high protein diets, or had been fasted three days. In those animals which had received little carbohydrate, the liver contained lower levels of organic acid-soluble phosphorus and did not form organic phosphates readily. Especially, the formation of adenosinetriphosphate after the administration of glucose was decreased. Since the administration of insulin increased the rate of incorporation of radioactive phosphate by the liver, and particularly the formation of the adenosine phosphates (50), the differences in the metabolism of phosphate and of carbohydrate by the livers of the rats on the various diets may have been partly the result of differences in the amounts of insulin available.

Further evidence of the effects of previous dietary habits on intermediate metabolism was presented by Tepperman *et al.* (51). If rats were trained to eat their full rations for each day in a period of a few hours, the increase in R.Q. after they were fed glucose was considerably higher than in control animals. Since in rats trained on a high carbohydrate diet and then given a plethora of glucose subcutaneously,



R.Q.'s of 1.3 to 1.4 were obtained regularly, the authors consider that fat formation from carbohydrate was increased in rate in these animals. Similar differences between trained and untrained rats persisted when they were functionally eviscerated, whether insulin was given or not. Evidence that the rate of formation of fat from glucose had been increased also in the livers of the trained rats was obtained by Dickerson *et al.* (52). The degree of saturation of the liver fatty acids was increased after glucose feeding in these animals but not in untrained animals, and the increase in R.Q. of isolated liver tissue when glucose was added to the medium was much greater in samples from trained rats than in those from control animals. No differences between trained and untrained rats in their metabolism of fructose was encountered in these experiments.

*Excretion.*—The maximum tubular reabsorption rate of glucose was increased by the administration of thyroid substance or of thyroxine to dogs (53). The effect was not due to increased blood flow, but, it was suggested, was the result mainly of increases in the rates of the active processes concerned in absorption.

Dominguez & Pomerene (54) have analyzed the kinetics of the disappearance of galactose from the plasma after rapid intravenous injection in dogs. On the assumption that the injected galactose diffused almost immediately into a certain volume which remained constant throughout the rest of the experiment, the authors were able to calculate the relationship of the rates of excretion and of utilization (disappearance by other means than excretion) to the plasma level of the sugar. The rate of excretion was found to be proportional to the plasma concentration, while the rate of utilization, though it increased with increasing plasma levels, appeared to approach a maximum. The amount excreted was, therefore, not proportional to the dose of sugar given, the fraction excreted being higher for larger doses. Eiler *et al.* (55) found that the rate of reabsorption of galactose by the renal tubules of dogs did not reach a maximum, but that the amount reabsorbed was a constant fraction of the amount filtered over a wide range of plasma galactose concentrations and of concentration ratios of creatine in urine and plasma. Although hyperthyroidism did not appear to increase the rate of reabsorption of galactose as it did that of glucose, the authors presented other arguments for the view that galactose also is transferred actively.

*Anoxia and shock.*—The effects on the blood sugar of rats of exposure to quite low oxygen tensions for various periods of time in a

variety of conditions were reported by van Middlesworth *et al.* (56). As others have found, an immediate but short lived hyperglycemia appeared to be the usual result of rapid induction of anoxia in normal animals not previously acclimatized. A moderate hypoglycemia usually developed later in fasted rats but not in well fed animals. Adrenalectomized rats, whether fasted or fed, became hypoglycemic without an initial increase in the blood sugar. Adrenomedullary stimulation might be expected as the first response to anoxia, and the variations in blood sugar changes with nutritive state would be explicable on the basis of corresponding variations in available amounts of liver glycogen. These authors also reported the failure of administered epinephrine to produce hyperglycemia in rats exposed to severe anoxia, even though the glycogen content of liver and muscle appeared normal; hence they suggested that normal glycogenolysis requires a continuing supply of oxygen. Such an explanation does not seem likely with respect to the breakdown of liver glycogen, which takes place readily when the liver is made anoxic either *in situ* or *in vitro*, but the formation of glucose from lactic acid by the liver might be expected to be interrupted in an anoxic state.

The hypoglycemic phase of response to anoxia may be the result of increased rates of removal of sugar from the blood in this condition, for partially hepatectomized rats, which could maintain normal blood sugar levels when at normal atmospheric pressures, developed marked hypoglycemia when exposed to severe anoxia (57). In eviscerated rats, the blood sugar level fell much more rapidly after hemorrhage than in control animals whether the adrenal medullae were present or not (39, 58). The amounts of lactate and of pyruvate in the blood and also the lactate-pyruvate ratios increased rapidly in adrenomedullated eviscerated rats which were subjected to hemorrhage, while there was little change in these values, except terminally, in control animals which were not bled (58). The suggestion is strong that after either hemorrhage or exposure to low oxygen pressures, anoxia of the tissues increases the rate of glycolysis as compared to the rate of oxidation of carbohydrate and in so doing increases the rate of carbohydrate utilization.

Anoxia of the liver of the rat, produced either *in vivo* after hemorrhage (59) or *in vitro*, was followed by a marked reduction in the oxygen consumption of the liver slices in the presence of adequate oxygen (60), apparently for the most part because of damage to the protein components of some of the enzyme systems (60, 61). Glucose had no

effect on the respiration of either normal or anoxic liver slices. The kidney of the rat proved much less susceptible to anoxia than the liver, and in experiments *in vitro*, it was protected to a considerable extent by the presence of glucose in the medium. Haist & Hamilton (62) reported almost complete failure of the liver to form glycogen from glucose in rats in shock following the release of clamps applied to the lower limbs. As long as the clamps remained in place, liver function appeared normal, and if the limbs were reclamped at a later time, considerable restoration of the ability of the liver to form glycogen followed. The authors considered that release from the anoxic tissue of some substance which affected the liver must have occurred, but whether a direct toxic factor was produced or a substance affecting nervous or hormonal control of carbohydrate metabolism, e.g., a substance stimulating epinephrine formation, was not evident. Severe atmospheric anoxia prevented liver glycogen formation in rats *in vivo* (62); and so also anoxia *in situ* diminished subsequent glycogen formation *in vitro* (63). Some of the decrease in glycogenesis which followed release of the clamps may have been the result of anoxia to the liver developing in this type of shock as it does after hemorrhage (59). Fluid loss was not believed to be a factor affecting liver function in these experiments, since the loss of fluid remained high in the animals in which the limbs were reclamped. However, the animals which were reclamped recovered from a state of shock which was otherwise fatal; so without further data on the state of oxygenation of the hepatic circulation and on other functions of the liver, the possible role of reduced circulation to the liver in lowering glycogen deposition cannot be evaluated.

In shock due to burns in human patients hyperglycemia and lactic acidemia commonly occur (64). Clark & Rossiter (65) found decreased muscle glycogen levels in rats and low liver glycogen in rabbits, and a marked decrease in the rate of glycogen synthesis by liver slices *in vitro* when the samples were taken from rabbits four hours after severe burns. Most of the effects of burns on carbohydrate metabolism appeared referable to stimulation of the adrenal medulla, although the authors believed that other mechanisms may also have been concerned. If shock sufficiently severe to produce hepatic anoxia occurred in these animals, the metabolism of the liver could have been affected directly; such a process would explain the discrepancy noted by these workers between the effects of burns and of administered epinephrine on the glycogen of the livers of rabbits.

## EXPERIMENTAL DIABETES AND THE INFLUENCE OF HORMONES

A number of papers have appeared in the last year or two concerning the nature of experimental diabetes and the factors controlling its development. Lukens (121) has reviewed the subject of the pathogenesis of diabetes. In summarizing his work on the prevention and alleviation of pancreatic damage after partial pancreatectomy, he puts forward the hypothesis that hyperglycemia is the chief causative factor in the subsequent failure of the pancreas and that with its prevention by phlorizin or insulin, the pancreas may be protected. This view may be considered complementary to that of Haist, who has reviewed the factors affecting the insulin content of the pancreas (119). Haist considers there to be two types of processes by which the amount of insulin in the pancreas may be reduced: those which reduce the need for insulin and so reduce its production, as fasting, low carbohydrate—high fat diets, and the administration of insulin; and those which increase the need for insulin relative to the available supply, as partial pancreatectomy or treatment with anterior pituitary extracts. In the latter type, permanent damage to the pancreas may be prevented by the giving of insulin. Another situation in which the need for insulin appears to be increased is that in which hyperphagia is induced by hypothalamic lesions in rats. Such lesions had no effect on a diabetes which was already established if the food intake remained unchanged; but in partially depancreatized (nondiabetic) rats, hyperphagia caused the appearance of severe diabetes (66).

Houssay (67) has presented new data on the relationship of thyroid activity to diabetes mellitus. He found, as have others, that thyroidectomy does not appreciably alleviate pancreatic diabetes in dogs, nor does the administration of thyroid hormone to normal dogs or rats produce diabetes. However, partially depancreatized (nondiabetic) dogs were quite sensitive to thyroid hormone, treatment with it producing temporary or permanent diabetes, and the diabetes of completely depancreatized dogs was made much more severe by its administration. It would appear that hyperthyroidism also leads to an increase in the need for insulin, and that this need may in turn lead to exhaustion of an already damaged pancreas. Stimulation of the thyroid may possibly be a factor in the production of the permanent type of diabetes with anterior pituitary hormones.

Stetten & Boxer (68) have utilized alloxan diabetes in order to study the rates of turnover of carbohydrate and fat in insulin deficiency. In a diabetic rat at constant weight on a high carbohydrate—low fat diet,

with deuterium in its body water, the rates of incorporation of stably bound deuterium into the urinary glucose, liver and carcass glycogen, and liver and carcass fat were determined. The rate of gluconeogenesis, as judged by the inclusion of deuterium in the glycogen, was above normal, but since the excreted glucose seemed to be largely of dietary origin, the rate of new formation of carbohydrate cannot have been excessively high. The rate of synthesis of fat was calculated to have been but a small fraction of the normal; the data support the suggestion made some years ago by Drury (69) that one of the principal effects of insulin is the promotion of the storage of dietary carbohydrate as fat.

In diabetic and normal dogs, the ratio of lactic acid to pyruvic acid in the blood was found to be constant over a wide range of concentrations (70); no evidence was found for any effect of insulin on the inter-conversion of lactic and pyruvic acids, but the failure of the blood pyruvate to increase after glucose administration in diabetic animals was confirmed.

*Alloxan diabetes.*—Since the discovery in 1943 that necrosis of the pancreatic islet cells and permanent diabetes mellitus may be produced by the administration of single doses of alloxan to rabbits and dogs, numerous papers have appeared reporting further investigations of this phenomenon. Alloxan diabetes has now been produced in the rabbit, rat (white and hooded), dog, and monkey (71), and specific pathological changes have been observed in the pancreas of the cat, guinea pig, and pigeon (72). Several detailed reports on the pathological and metabolic changes obtained in the rabbit, dog, and rat are now available (72 to 79). In the typical experiment, three stages of response to a single dose of alloxan are apparent: an immediate hyperglycemia reaching a maximum in two or three hours; then a hypoglycemic phase lasting for several hours, often accompanied by convulsions and frequently fatal unless relieved by glucose; and finally an increase in the blood sugar to levels above normal, with glycosuria evident by the first or second day. The responses of individual animals to given doses of alloxan vary considerably, however, so that either the initial hyperglycemic or the hypoglycemic phase may not be pronounced, and the diabetic phase may vary in severity from a mild transient type to a progressive fatal condition. With large doses of alloxan, damage to the liver and kidney may be produced (72, 74, 78), but with the minimal effective diabetogenic doses, there is usually little or no extra-pancreatic injury. The insulin content of the pancreas is quite low in

diabetic animals (77, 80). In most respects the diabetes resembles that following partial or complete pancreatectomy. The glucose tolerance is reduced during the diabetic stage, and the glycosuria may be controlled with insulin; it may be reduced or in some cases abolished by a diet high in fat, and it also falls with diminished food intake (81). The glycogen content of the liver and skeletal muscle is reduced, that of the heart increased (82). Ketonuria is common, and late cataracts have been observed (83). In animals already diabetic following pancreatectomy or previous alloxan administration, the hypoglycemic effect of alloxan was not observed (77, 78). Partially depancreatized rats have been reported to be relatively resistant to the diabetogenic action of a single dose of alloxan (84). Small repeated doses of alloxan are as effective as single large doses in producing diabetes in both normal and partially depancreatized animals (78, 83, 84, 85), and they appear to be less toxic.

Alterations in the islet cells after the administration of alloxan appear to be confined to the  $\beta$  cells, and they are exclusively degenerative in nature (73, 76). Since some changes may be observed in as short a time as five minutes after the administration of alloxan (73, 86) and since necrosis is quite advanced by the end of a few hours, the hypoglycemic phase of alloxan action can hardly be the result of stimulation of the pancreas. This conclusion is supported by the observations of Ridout *et al.* (80) that the insulin content of the pancreas falls only with the onset of hypoglycemia or later, at a time when most of the cells appear to be already dead. The hypoglycemia is adequately explained by the leaching of preformed insulin from the disintegrating islet cells (86).

The initial hyperglycemic effect of alloxan may be due to adreno-medullary activity. Goldner & Gomori (77) did not observe this phase in completely adrenalectomized or adreno-demedullated (formalin injected) rabbits. Ergotoxin was reported in one experiment to prevent the hyperglycemia (87), and histological changes considered indicative of stimulation of the medulla have been observed (88). The data on this point seem rather scanty, however, and some extra-medullary process may yet prove to play a part in the production of the initial hyperglycemic phase. In any case, the prevention of hyperglycemia by insulin or phlorizin does not interfere with the subsequent development of permanent diabetes (77), so that damage to the pancreas following hyperglycemia cannot be an important factor in the production of diabetes by alloxan.

The reason for the specific necrotic effect of alloxan on the cells of the pancreatic islets is unknown. Alloxan does not inactivate insulin either *in vitro* or *in vivo* (77, 78). Although alloxan is known to inactivate many enzymes by oxidation of their sulfhydryl groups, rather high concentrations (about 0.01 *M*) are required for this action; and since the administration of other oxidizing agents of the same or greater power does not produce diabetes (77), this property of alloxan would not seem to be the basis of its action on the pancreas. Alloxan in low concentration may act as hydrogen acceptor for certain dehydrogenases (89, 90); it seems not unlikely, therefore, that some specific enzyme in the  $\beta$  cells may have a high affinity for alloxan and be inactivated by union with it. A large number of compounds related in structure to alloxan have been tested for their diabetogenic effect (77, 91). Of these, only alloxantin (formed by the union of alloxan with its primary reduction product, dialuric acid) was said in one report to be effective (92). Since the results were negative in other tests of this substance (77), this point requires further investigation.

*Anterior pituitary.*—Young has given an account of the effects of the chronic administration of anterior pituitary extract (unfractionated) to growing dogs (93). Only after some months of treatment, as the dogs ceased to grow, did diabetes appear. With the control of the diabetes with insulin, continued treatment with the extract appeared to induce further growth as judged by increases in weight and nitrogen retention. During the administration of the extract (diabetic phase), the animals were quite resistant to insulin. This fact suggests that during the nondiabetic (growing) stage of treatment there may have been an increased strain upon the pancreas which led ultimately to its insufficiency in the grown animals. These results are consistent with the views advanced previously by others that some insulin must be present for the induction of growth by pituitary hormone and that in its absence diabetogenic effects only may be observed. However, until pure (one component) preparations of growth hormone are available for use in completely depancreatized animals, it cannot be ascertained whether these two effects are different aspects of the activity of one hormone or are in fact due to the action of two separate substances.

Ogilvie (94) attempted to induce a permanent pituitary diabetes in rabbits, like that demonstrable in dogs. However, although glycosuria occurred in most of the animals, it lasted on the average for only ten days, and the glucose tolerance and insulin sensitivity, which were reduced during the diabetic phase, returned to normal later. Follow-

ing prolonged treatment with anterior pituitary extract, considerable hypertrophy of the pancreatic islets was demonstrable in these animals. In rabbits as in normal rats, the pancreas is apparently able by hypertrophy to overcome the effects of pituitary extract; whereas in the adult dog or in partially depancreatized animals the pancreas seems to become exhausted by its increased activity and often to suffer permanent damage. Since the differences in response of these two types of animals may lie only in differences in the ability of the islet cells to hypertrophy in response to needs, the existence of a separate pancreatic factor cannot be said to have been demonstrated.

Preparations of various anterior pituitary hormones in highly purified form have been studied with respect to their ability to maintain the muscle glycogen levels in fasted hypophysectomized rats (95). The results were substantially the same as those obtained previously in the same laboratory with relatively crude preparations: the glycostatic effect was greatest in growth hormone preparations, but its concentration did not parallel growth promoting activity during purification; purified lactogenic, thyrotropic, and adrenotropic hormones had little or no activity when given in the usual twenty-four hours test; adrenotropic hormone alone, when given over several days, was found to maintain not only muscle glycogen but also liver glycogen and blood sugar levels as well. The purified growth hormone, like crude extracts, has a hypoglycemic effect on fasted hypophysectomized rats (96). The administration of unfractionated anterior pituitary extract to rats twenty-four hours before the experiment prevented the usual effect of insulin *in vitro* on the formation of glycogen by isolated diaphragm segments (97).

Houssay *et al.* attempted to determine the glucose requirements of eviscerated dogs after hypophysectomy, pancreatectomy, anterior pituitary extract or thyroid treatment, or without other treatment (98). Although a considerable number of animals was used and unsatisfactory preparations were discarded, wide variations in the apparent glucose requirements as well as in the changes in muscle glycogen and blood lactate made it impossible to calculate satisfactory figures for total carbohydrate utilization in these preparations. No significant differences between the glucose requirements of the several series of an animal could be distinguished.

*Adrenal cortex.*—Whether the adrenal cortical hormones affect the rate of phosphorylation in tissues is still a matter of controversy. Montigel & Verzar (99) have published further data illustrating an



apparent decrease in the rate of phosphorylation in muscle and liver tissue from adrenalectomized cats, the rate of disappearance of inorganic phosphate being determined in finely minced tissues diluted with bicarbonate buffer containing sodium fluoride and added glycogen. The exact nature of the limiting factor in these experiments was not determined. Since the adrenalectomized cats were in states of severe adrenal insufficiency and the animals were in addition exsanguinated before the samples were taken, it seems possible that changes not specifically related to lack of the adrenal hormones may have taken place in the tissues, e.g., destruction of enzymes or cofactors as a result of prolonged partial anoxia. Shapiro & Wertheimer (100) observed no change in the rate of phosphorolysis of glycogen in muscle mince from adrenalectomized rats which had been maintained in good condition with salt. However, Montigel & Verzar also report restoration of normal rates of phosphate disappearance in muscle mince after the addition *in vitro* of pure cortical hormones (desoxycorticosterone, 0.01 per cent or higher, other steroids in greater concentrations). Should these results be confirmed, further investigations of the reactions affected would be interesting. In another paper the same authors report increased deposition of glycogen in the liver of adrenalectomized cats treated with desoxycorticosterone, but the figures presented do not appear to differ significantly from the normal (101). The low serum amylase levels which these authors also observed in cats in adrenal insufficiency were correlated with diminished food intake (102).

Adrenalectomy or gonadectomy did not affect the insulin content of the pancreas, nor influence the usual changes in insulin content found in response to alterations in diet (103). Some increase in the insulin content of the pancreas of the hypophysectomized rat following treatment with adrenotropic or lactogenic hormone was reported (104).

Ingle (105) found the diabetogenic response to stilbestrol to be demonstrable in adrenalectomized hypophysectomized partially depancreatized rats, although its action was much less marked than in control depancreatized animals. The animals were maintained during the experiment on subdiabetogenic amounts of adrenal and pituitary hormones, and Ingle had previously found that for stilbestrol to induce diabetes in the absence of the adrenals, some cortical hormone had to be supplied. The nature of the effect of stilbestrol on carbohydrate metabolism is still obscure; but it appears that in this case, as in a number of others—the production of diabetes in depancreatized

rats and the lowering of the respiratory quotient in glucose-fed rats by anterior pituitary extracts, and the increase in glycogen deposition in response to low atmospheric oxygen—some cortical hormone but not the intact adrenal gland needs to be present.

### METHODS

A number of improved methods for the determination of sugars in biological materials have been proposed. Nelson (106) has adapted the Somogyi copper reduction method to photoelectric colorimetry by using with it a new arsenomolybdate color reagent. The use of dinitrosalicylic acid as a reagent for determining blood sugar has been described by Sumner & Sisler (107). Manometric measurement of the carbon dioxide produced by yeast fermentation of glucose proved a satisfactory quantitative method when sodium azide was added to the medium to prevent assimilatory reactions (108). The Conway micro-diffusion technique was used by O'Malley *et al.* (109) in the direct fermentative measurement of blood glucose. Morris (110) determined glucose and maltose in mixtures by taking advantage of the differential reduction of alkaline copper and of ferricyanide reagents by the two sugars. Haas (111) estimated glucose-6-phosphate by following colorimetrically the reduction of 2-6-dichlorophenolindophenol by the hexose ester in the presence of suitable enzyme and coenzyme preparations. Improved reagents for the removal of nonsugar reducing substances from urine were described by Dittebrandt, Tenney & West (112).

Methods for the preparation and purification of glucose-1-phosphate have been described by McReady & Hassid (113) and by Sumner & Somers (114).

New data on the effects of exercise (115) and of atmospheric temperature (116) upon the response of rabbits to insulin emphasize again the necessity of rigid control of experimental conditions for the assay of insulin and for the estimation of insulin sensitivity.

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## FAT METABOLISM

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This review covers some aspects of fat metabolism which have not been discussed in detail in previous years. It has been difficult to obtain foreign literature and therefore the review is incomplete.

### NERVOUS SYSTEM AND FAT METABOLISM

*Relation of the hypothalamus to fat metabolism.*—Since 1940 when the symposium on the hypothalamus was published by the Association for Research in Nervous and Mental Diseases (1), many groups of investigators have made systematic attacks on the problem of hypothalamic regulation of metabolism including that of fat. Hetherington & Ranson (2, 3, 4) have demonstrated conclusively in rats that carefully placed bilateral lesions in the ventromedial nuclei of the hypothalamus (injury to pituitary was avoided) produced promptly increase of appetite and subsequently extreme obesity. Removal of the pituitary provoked no obesity, and also did not prevent development of obesity following hypothalamic lesions. Even when sufficient time after hypophysectomy, eleven weeks, was allowed to elapse for atrophy of thyroid, adrenals, and gonads to develop, subsequent hypothalamic lesions were still followed by hyperphagia and obesity. These results have been confirmed in the monkey by Brooks, Lambert & Bard (5), in the dog by Heinbecker *et al.* (6), and in the cat by Wheatley (7).

Hetherington (4) has tried, in an extensive series of experiments, to localize the lesions which consistently result in obesity. He concluded that cell groups of basal forebrain rostral to the ventromedial hypothalamic nucleus made little if any contribution to regulation of fat metabolism. Destruction of the ventromedial hypothalamic nucleus or its descending fibers in the brain stem was required to produce hyperphagia and obesity.

That diffuse injuries to brain may disturb fat metabolism has been suggested by the effects of insufflation of air into ventricles of the dog. Schrader (8) observed that three to four hours after air injection the total blood fats were reduced in amount. Phospholipids, cholesterol, and neutral fats all decreased in concentration. The change in cho-



lesterol occurred almost exclusively in the ester fraction. It should be mentioned that other workers have found no such effect in man (9).

Brobeck, Tepperman & Long (10, 11) have investigated the energy metabolism of rats and of pair fed controls with experimental hypothalamic hyperphagia and obesity. Metabolic effects, including rise in R.Q. were found to be secondary to the hyperphagia and obesity. The distribution of enormous fat deposits appeared normal.

Brooks & Bard (12) have also found a rise in R.Q. above unity in monkeys and rats with hyperphagia following production of a hypothalamic lesion. The contention, however, of Brobeck, Tepperman & Long that the method of eating accounted satisfactorily for the rise in R.Q. is open to question. Brooks & Bard have designed experiments which should settle this question but at this stage of their work they are unwilling to draw conclusions.

It is noteworthy that in all of these papers, except for a statement by Hetherington that special stains might reveal lesions and for Heinbecker's observations, no mention was made of any changes occurring in the anterior pituitary. In fact it has been repeatedly demonstrated that severance of the pituitary stalk produces no change in the anterior pituitary. Westman and co-workers (13) have reinvestigated this problem of diencephalo-hypophysial relations. They performed the following experiments in rats: (a) Separation of the hypophysis from its infundibulum and the surrounding pars tuberalis, (b) extirpation of the processus infundibuli, and (c) separation of the processus infundibuli from the infundibulum. In the animals of group (a) the pars distalis showed noticeable microscopic changes. The cells were uniform, and the alpha cells were absent. The genital organs exhibited considerable atrophy. In the other two groups of animals, in which the connection between the pars tuberalis and the pars distalis was left intact, the pars distalis and the genital organs were normal. The authors emphasized that the connection between the hypothalamus and the hypophysis consisted of two different parts. The one consists of the pars tuberalis with the portal vessels and a specific nerve fiber system; the other is formed by nerve tracts that pass from certain hypothalamic nuclei to the processus infundibuli and to the pars intermedia. Heinbecker *et al.* (6), like previous workers, did not find that stalk section (separation of the hypophysis from the infundibulum and pars tuberalis) produced any changes in anterior pituitary such as those described by Westman. In addition to making small paraventricular lesions in the hypothalamus of dogs, Heinbecker and co-workers (6)

destroyed most of the supraoptic as well as the paraventricular nuclei in many animals. These workers observed that normal dogs when caged and fed unrestricted diets tended to become moderately obese more frequently than did rats or monkeys. Some of their hypophysectomized animals became fat. Lesions in the caudal portion of the paraventricular nuclei however produced marked obesity, but the most marked obesity occurred when the supraoptic nuclei were also destroyed. This later group of animals was remarkable in a number of other respects. There was marked loss of basophil cells in the anterior lobe of the pituitary, atrophy of thyroid and gonads, and irregular changes in the islet cells of the pancreas. The adrenals did not atrophy. Except for the loss of basophil cells the anterior pituitary was not remarkable on histological examination. Adrenal cortical extract when given subcutaneously in daily doses of 4 to 8 cc. increased the dogs' blood plasma cholesterol from an average of 194 to an average of 315 mg. per cent. Similar doses had no effect in normal dogs. Heinbecker (14) utilized these data in an investigation of the pathogenesis of Cushing's syndrome. He concluded that in addition to tumors of the adrenal cortex or of the thymus, atrophy of the nuclei of the hypothalamus should be considered as a possible precursor of the hyalinization or other changes of the basophil cells of the anterior pituitary. Heinbecker suggested that destruction of certain hypothalamic nuclei increases the effectiveness of the adrenal cortical hormone and in this way the changes in basophil cells may be explained.

In spite of the excellent work on hypothalamic and hypophysial relations, the mechanism whereby the nervous system regulates the anterior pituitary remains unsolved. It now appears that many organs besides the endocrines, such as the kidneys, intestines, and liver, may be seriously affected by injury to the stalk and hypothalamus. The experience of Graef, Negrin & Page (15) of finding cirrhotic livers in some dogs after hypophysectomy led them to check the hypothalamus. They found lesions in the hypothalamus of the dogs with cirrhosis. They now suggest this combined lesion as a factor in the production of liver cirrhosis in dogs after hypophysectomy and thyroidectomy as described by Chaikoff *et al.* (16). Much of the confusion regarding experimental results arises from failure of many workers to check by histological methods both the hypothalamus and the pituitary.

Conn (17) has reviewed critically and comprehensively investigations into the etiology of obesity. He pointed out that conclusive as the experiments on hypothalamic obesity in animals may be, such lesions

have only rarely been demonstrated as a cause of obesity in man (14). He concluded that most cases of obesity arise from environmental experiences, conditioning, habits, and psychological attitudes toward food. Newburgh (18) has summarized the work on energy metabolism in obesity. These data support his already well-known contention that control of obesity depends on control of appetite. Other less comprehensive articles have been published during the year (19 to 24).

*Effect of sex hormones on the chemical constituents of brain.*—Weil (25 to 28) has presented evidence that there is a correlation between sex of the animal and the chemical constitution of the brain. The phosphorus content of alcoholic extracts of brain from sexually mature rats was higher with female than with male brain. The difference appeared to be most marked in the phosphorus content of the cephalin fraction of brain tissue extracts. By injecting androgenic or estrogenic hormones into young rats (27, 28) it was possible to produce changes in the phosphorus content of the cephalin fraction. Androsterone injected into twenty-five day old female rats reduced the phosphorus content to that of male brain tissue. Estrogen injected in male rats resulted in corresponding reversal. Homologous glandular substance had no effect. Castrated animals developed no change in phosphorus content of the cephalin fraction with age. The injection of sex hormones in castrated or spayed rats reproduced transformations that occur normally.

In connection with Weil's reports the histological studies made by Vazquez-Lopez (29) are of interest. Prolonged treatment of the hamster with estrogens produced striking changes in cells of the pituitary and the hypothalamic nuclei. Proliferation of cells occurred in the pars nervosa, but the anterior pituitary was not affected until late. The cells of the supraoptic nucleus became vacuolated and disappeared. According to data reported by Cardini & Serantes (30), stilbestrol reduced tissue phospholipids of all organs except brain and kidneys of normal female rats. The blood plasma phospholipid content remained unchanged. The action in spayed rats was similar but less marked than in intact animals.

#### ENDOCRINES AND FAT METABOLISM

The effect of the various endocrine glands and their products on fat metabolism continues to be a controversial subject.

*Pituitary and adrenal glands.*—Sayers *et al.* (31) have presented new data indicating that adrenotropic hormone when injected in rats

reduced adrenal cholesterol. From these findings and a critical review of the literature the authors explained many of the previously conflicting results. They described five degrees of adrenal response to stress: (a) Sudden temporary stress which results in temporarily and partially depleted adrenal cholesterol; (b) slow change in environment, which produces no change in cholesterol; (c) severe stress ending in death, which markedly depletes adrenal cholesterol; (d) hypersecretion of pituitary when there is little use for cortical hormone, which results in storage of adrenal cholesterol; (e) loss of pituitary, which leaves adrenal cholesterol inert and more responsive to changes in internal and external environment. Abelin (32) has reported that ingestion of glucose reduced adrenal cholesterol. This effect was modified by administration of corticosterone of anterior pituitary extract and of thyroid.

Conn (17) pointed out that little experimental or clinical evidence remains to support the diagnosis of pituitary obesity. Dysfunction of hypothalamic centers was required for development of the adiposogenital dystrophy. He suggested that increased fat deposition may delay gonadal development. The genital hypoplasia, on the other hand, may be explained by Heinbecker's experiments (6) which were discussed in the section on nervous system and fat metabolism. The effects of pituitary dysfunction, therefore, are usually manifested through its control of the thyroid, adrenals, and gonads. Levin (33) demonstrated that hypophysectomized rats could be restored to normal weight by forced feeding. Analysis of the tissues of the rats for fats, protein, and water content showed that the increased weight was the result of fat deposition.

Conn (17) demonstrated that the bull-like neck in patients with Cushing's disease was due to a large deposit of fat, but that the large abdomen was not caused by increase in fat but was due to lax muscles. The total weight of patients' adipose tissue may be below normal. Caloric restriction resulted in loss of weight but no improvement in distribution of fat. The basal metabolism, although frequently low, was variable. Data on detailed analyses of adrenal cortical substances continue to be reported by Prelog, Ruzicka & Weiland (34, 35) and also by Reichstein and others (36, 37).

Swingle & Remington (38) concluded from a review on the effects of adrenalectomy that evidence is insufficient to justify suspicion that disturbed fat absorption plays a role in metabolic upsets following adrenalectomy. The most recent investigations of this problem yield

conflicting results (39). The effects of adrenalectomy on the mobilization of fat in rats on diets high in butter fats or high in tung oil, and finally while fasting were studied by Samuels & Conant (40). The diets containing tung oil increased excretion of acetone bodies in all animals. Excretion of acetone bodies was always greater in normal fasting rats than in adrenalectomized rats on the same diet. Liver fats of adrenalectomized and control rats on tung oil diets fell rapidly during fasting. The fall was not as prompt as in animals on butter fat diets. Adrenalectomized rats used depot fats at a slower rate than controls. These authors interpreted their results as indicating disturbance in transport of fats after adrenalectomy rather than any change in fat absorption. Bavetta (41) tested effects of adrenalectomy on absorption of short chain and long chain acids. The first indication of delay was found for caprylic acid, shorter chain acids were not affected. He concluded that adrenal glands play an active role in absorption of long chain acids.

*Gonads.*—Sperry and co-workers (42) found that thyroidectomy and castration in monkeys appeared to have no effect on serum cholesterol. Hard and co-workers (43) analyzed for carbohydrates, fat, and water in the liver, blood, and placenta of pregnant, fetal, and newborn guinea pigs. Fetal liver fat concentration rose during the last quarter of gestation and decreased after birth. The R.Q. dropped from 1.0 at birth to 0.63 at twelve and thirteen hours, then rose to 0.8 or above after fifteen days. Incidental to studies on the nutritive value of diets Deuel and co-workers (44) observed that the lipid content, as well as the ash and calcium content, of tissues of male rats was lower than that of female rats. These differences were remarkably constant in a series of one hundred and two animals.

*Thyroid.*—As in previous years it was demonstrated that thyroidectomy had little or variable effect on the lipid metabolism of monkeys (42) and dogs (45). In man, lack of thyroid resulted in marked increase in blood serum lipids. Excess of thyroid tended to reduce serum lipids but to a variable extent (46). Forbes (47) reported that thyroxine reduced neutral fats in carcasses and livers of rats living on diets that were high in carbohydrate, low in protein, free of fat, and moderate in choline content. When choline was omitted liver fat was high.

#### FACTORS AFFECTING BLOOD AND TISSUE FATS

Many of the conflicting reports in the literature on fat metabolism arise from neglect of species and individual differences, time factors,

and diets. The method of studying fat metabolism in diseases in man has depended largely on the determination of lipids in blood serum or plasma. Serum lipid analyses have been difficult to interpret because in severe cases of disease the ingestion of food may be minimal and the effects of starvation and dehydration on lipid metabolism have not been clear.

*Starvation.*—Kartin *et al.* (48) have studied the effects of starvation for two to six days in fourteen healthy young men, eleven of whom were simultaneously restricted or deprived of water. In these men, although blood ketones increased progressively, neutral fat changes were equivocal and serum total cholesterol was not significantly increased until starvation was prolonged more than two days. After three to six days the average increase in serum total cholesterol was  $36 \pm 13$  mg. per cent. Lipid phosphorus rose perceptibly but exceeded the range of normal variability ( $\pm 0.7$  mg. per cent) on only seven of twenty-one occasions. Administration of sufficient carbohydrate to mitigate or extinguish the ketosis abolished the hyperlipemia. The blood ketones and serum lipids of four male rhesus monkeys (*macaca mulatta*) which were starved for three to six days rose more rapidly and further than those of men. The monkeys were allowed water *ad libitum*. Again the neutral fat did not change significantly although the total cholesterol increased by an average increment of 58 mg. per cent or 49 per cent of the initial value. The rise was chiefly in the esterified fraction with the result that the ratio of free to total cholesterol fell. Sperry, Jailer & Engle (42) have demonstrated that the monkey does not maintain the proportion between the cholesterol fractions as constantly as does man, so that this fall in per cent of free cholesterol may be attributable to the species used rather than to the effect of starvation. Fat metabolism in other species will be discussed later. The serum lipid phosphorus of the monkeys studied by Kartin *et al.* (48), while less affected than cholesterol, increased by an average of 2.4 mg. per cent or 30 per cent of the initial value. Ingestion by the monkeys of 15 to 20 gm. of sugar daily for from three to seven days diminished ketonemia and prevented the rise of lipid phosphorus but not of cholesterol. When 40 to 50 gm. of sugar were given daily ketonemia was abolished and serum cholesterol fell to or below its initial concentration. Seven dogs, which were starved from four to fourteen days and which, with two exceptions, were given water *ad libitum*, developed neither appreciable ketosis nor hyperlipemia. It was suggested that the hyperlipemia of carbohydrate starvation arises not

merely because a greater amount of fat is being utilized but because an unusually large amount of fat is being converted to ketone bodies.

*Intake and synthesis of lipids.*—That concentration of lipids in various fractions of blood is little influenced by variations in fat intake has been demonstrated in man by Oppenheim & Bruger (49) and Gough (50), and in dogs by Miller & Hansen (51) and Izzo & Marenzi (52). Even after large doses of insulin, Kaplan, Entenman & Chaikoff (53) noted that the blood lipids of man did not change appreciably. Steiner & Domanski (54) fed soya lecithin to normal men for six weeks. Serum cholesterol was lowered significantly for five weeks and then returned to normal despite continuance of diet. The constancy of serum lipids is in accordance with the observations of Reinhardt, Fishler & Chaikoff (55) that labeled phospholipids injected into the blood stream of dogs disappeared rapidly from the plasma. In contrast to these short term effects, it must be remembered that long periods of malnutrition may markedly deplete blood serum lipids in man (56).

That species differences in composition and distribution of fat exist is well known but no thorough investigation has ever been made of this problem. More data have been added by Boyd (57), who employed a modification of Bloor's oxidation method for the analysis of blood plasma and red cells from 10 guinea pigs, 10 bull frogs, 116 albino rats, 89 rabbits, 3 cows, 27 cats, 22 cockerels, 118 men, and 18 dogs. The dog had the highest concentration of plasma lipids of the group, the guinea pig and the bull frog had the lowest. The bull frog had no neutral fat. The standard deviation for most lipid fractions was highest in the dog. Analyses of red cells separated from oxalated blood were also given. Boyd found that the percentage relations between component lipid fractions were comparatively constant from species to species.

Frazer (65) has investigated the problem of fat absorption. He has compared the distribution of fat after feeding rats olive oil alone or olive oil and lipase. When the lipase was added there was a portal rather than a systemic lipemia, and fatty deposition occurred in the liver rather than in the usual fat depots. In humans, by means of the chylomicron technique, it was shown that the lipemia following the ingestion of 30 gm. of fat was greatly diminished or prevented when lipase was given. Frazer concluded that glycerides could be absorbed without hydrolysis into fatty acids but that the processes of absorption and the immediate deposition of free fatty acids and of glycerides

differ. In two later articles Elkes & Frazer and others (66, 67) have investigated the role of emulsification and of phospholipids in fat absorption.

MacLachlan (71) has observed the effect of fasting for one to four days on the phospholipids, total lipids, acetone-soluble lipids and the iodine numbers of the phospholipids and the acetone-soluble lipids in the blood of fasted mice. In the monkey Sperry and his co-workers (42) found that the serum cholesterol concentration did not increase during eight hours immediately following, and at a twenty-four hour period after the ingestion of eggs. Serum cholesterol increased slightly when cholesterol, either in the form of eggs or as cholesterol in oil solution, was given for a longer time, four days.

Barnes, Primrose & Burr (69) found a greater digestibility of fat by rats which received a diet containing 30 per cent protein than by rats which received a diet containing 14 per cent protein. Samuels & Conant (40) noted a decrease in liver fat during six days of fasting of young male rats previously fed a high fat diet. Nutritional values of fats in various species have been investigated (44, 58 to 64).

As a step in the investigation of the problem of ketosis, Morehouse (68) found that tributyrin was not converted directly into long chain fats. There was only temporary storage of the butyrate molecule but in what form it was stored was not ascertained. Bullet & Bernhard (70) compared the formation of oleic and saturated fatty acids in rats fed fat free bread and given water containing deuterium. Other work has been done on fat metabolism and ketones by use of labeled compounds (72, 73, 74). Block & Rittenberg presented evidence to support the view that acetate is a specific cholesterol precursor (75) and to demonstrate the synthesis of fatty acids from acetic acid (76).

The above investigations have shown that the living animal has an amazing capacity for maintaining a constant level of serum lipids in spite of fasting and of marked variations in the amount and kinds of fat ingested; that the synthesis of fatty acids is an active process; and that analyses of tissues for different lipids and ketone bodies are necessary to understand the elaborate dynamic chemical changes of fat metabolism.

*Liver.*—Peters, Kartin & Man have presented data on the interrelation of serum lipids in 68 patients with liver disease (77, 78). These data were supplemented with a few observations on the use of choline chloride in human liver disease and on the liver lipids of nine patients, with a variety of syndromes, whose serum lipids before death



had been measured (79). These investigations, as well as those of Ralli (80), have confirmed earlier observations that the most frequent disorder of serum lipids in liver disease is an increase of the ratio of free to total cholesterol, but Peters *et al.* have pointed out that this alteration is frequently due not only to an absolute increase in free cholesterol but to a deficiency in esterified cholesterol. Especially in subjects with liver disorders attended by hyperlipemia, phospholipids increased proportionately more than total cholesterol, making the ratio of lipid phosphorus to cholesterol greater than in normal subjects. The greatest hyperlipemia occurred in patients with biliary obstruction. Subsequent to operative relief the serum lipids fell. The serum lipids were also elevated, though not to as great an extent, in certain patients with biliary cirrhosis and infectious hepatitis. The latter patients usually had acholic stools and intense jaundice at the onset of the illness. This suggests that hyperlipemia is a product of biliary obstruction. In most patients with portal cirrhosis, cholesterol and lipid phosphorus were normal or subnormal and tended to fall as the disease advanced. In patients with toxic hepatitis and some with infectious hepatitis, cholesterol and phospholipids were also reduced. The hypolipemia seemed to be associated with extensive degeneration or destruction of liver parenchyma. In liver disease neutral fat seldom rose above the upper normal limits, although it was elevated in the acute stage of obstructive jaundice and of infectious hepatitis in certain patients. In six of Ralli's eighteen cirrhotic patients plasma neutral fat was increased, and, in five of these six patients, plasma total cholesterol was elevated (80).

Administration of choline chloride to eight patients did not seem to alter the course of the serum lipid concentrations or of the disease in portal cirrhosis or infectious hepatitis (79). In those patients who improved, it was impossible to ascertain whether the choline chloride was instrumental in accelerating the recovery process. It might be inserted here that previous work on the effect of choline on lipids in blood or serum seems to have been confined to the effect of cholesterolemia in rabbits. Choline did not reduce the hypercholesterolemia of cholesterol fed rabbits (81).

Serum, total, and esterified cholesterol were determined in a few cases of hepatitis by Neeffe *et al.* (82) but the results on their control subject were so abnormal that it is impossible to compare their data with usual data on serum cholesterol. Turner *et al.* (83) reported that methionine and choline did not influence the disease pattern in soldiers acutely ill with hepatitis after inoculation with yellow fever

vaccine. Beattie and co-workers (84) found that methionine seemed beneficial in a single case of toxic hepatitis occurring after carbon tetrachloride poisoning. Echaurren & Jorquera (85) have reported that in seven of ten cases of cirrhosis the daily administration of 600 mg. of inositol combined with a high protein diet resulted in definite subjective and clinical improvement.

Sellards & McCann (86) have reported that after choline chloride was given orally to eight of fourteen rhesus monkeys inoculated with yellow fever five of the treated monkeys survived but three of the treated and all six of the monkeys untreated with choline chloride died. The livers of the untreated animals showed more disorganization and acute liver destruction than those of the treated monkeys. Findlay, Martin & Mitchell (87) have compared the clinical and pathological similarity of infectious hepatitis and post-inoculation jaundice, especially in regard to yellow fever. It is difficult to draw any conclusions from these experiments on monkeys in relation to liver disease in human patients because there are variations from species to species in the development of experimental hepatic injury and in the lipotropic action of various agents (88).

Lipid analyses on livers from human subjects have been made by various investigators. Man and others (79) found extremely high neutral fat, total cholesterol and cholesterol esters, and a low phospholipid in the liver of only one of nine miscellaneous cases. The patient had been on a protein deficient diet and had also taken rubbing alcohol by mouth. Ralli (80), in a series of eighteen cirrhotic patients, found the neutral fat increased in five of the livers analyzed at autopsy. In three of these five, plasma fatty acids *ante mortem* had been elevated. In four of the five the elevation in neutral fat was accompanied by an increase in liver total cholesterol. Bürger & Plötner (89) have reported that lipids of liver are lower in unsaponifiable material than in cancerous tissue from the same liver. Abels *et al.* (90) by use of liver biopsy found that the preoperative administration of 8 gm. of lipocaic to patients with gastrointestinal cancer and to patients with benign gastrointestinal disorders resulted in an average liver total lipid content lower than in fasted preoperative subjects. This reduction did not always occur in patients with obstruction of the common bile duct. The preoperative administration of five doses of 240 mg. of inositol resulted in an even greater reduction of liver total lipids. Preoperative treatment with 3 gm. of choline chloride reduced the liver total lipids, but not to as great a degree as treatment with inositol. Preoperative ther-

apy with 250 gm. of glucose diminished the liver total lipids. No fractionation of liver lipids was done, hence it is not possible to know whether glucose and lipotropic agents exerted the same type of reduction. That fractionation of the lipids is necessary to ascertain the type of fatty liver is emphasized by study of the extensive review of McHenry & Patterson (91), who have described the various types of fatty livers in experimental animals. Canman (92) found the liver lipids normal in a case of Neimann-Pick disease.

Many articles on the liver and on liver disease were published this year, but did not contain relevant data on fat metabolism in liver disease. Experimental work on the liver has been reviewed by Mann (93, 94).

In the field of animal experiments, Montgomery, Entenman & Chaikoff (95, 96, 97) have published a series of articles on antifatty liver factors from the pancreas as tested in depancreatized dogs maintained with insulin. Dragstedt's preparation from the pancreas called "lipocaic" was found to be a poor source of the pancreatic antifatty liver factor. In addition to the development of fatty livers, this preparation did not prevent a fall in cholesterol, phospholipids, or total fatty acids of the blood below preoperative levels (96). The last paper of this series (97) gave the method of preparation of the antifatty liver fraction by precipitation with ammonium sulfate from dilute acid extracts of pancreas.

The effect on the guinea pig of a diet high in cholesterol has been studied by Okey (98) in relation to the liver, lungs, adrenals, heart, and blood vessels, blood and bone marrow and anemia. The cholesterol feeding quickly produced a striking increase in esterified cholesterol and neutral fat and a decrease in lecithin in the liver. These changes were slow to disappear after the large amount of cholesterol was omitted from the diet.

Fishler *et al.* (99) from experiments on hepatectomized dogs given radioactive phosphorus concluded that plasma phospholipids were derived mainly from the liver. Later, in the same laboratory, a rapid transfer of plasma phospholipids to thoracic duct lymph was demonstrated (55). Patterson, Keevil & McHenry (100) observed increased phospholipid turnover in both the liver and kidney of rats when choline was added to the basal diet. They measured the percentage of administered radioactive phosphorus in the phospholipids in these organs. Boxer & Stetten (101) considered that the development of fatty livers is related to the rate of turnover of choline phospholipids even though the actual quantities of choline phospholipids in the body

may remain more or less constant. Later Stetten *et al.* (102) have published experiments from which it was concluded that the fat in fatty livers after choline deficiency owed its origin to inhibition of the transfer of fat from the liver to the depots. Fishman & Artom (103, 104) have continued their investigation on the effect of choline, choline precursors, vitamin B, amino acids and their combinations on the concentration of choline and noncholine phospholipids in the livers of young rats of different ages. Kaucher *et al.* (105) determined the amounts of different phospholipids in various tissues including beef liver. Riley (106), from specific activities of lecithin, cephalin, and sphingomyelin phosphorus in liver, kidney, and lungs of rats after administration of phosphorylcholine containing radioactive phosphorus could find no evidence "that phosphorylcholine as a unit is utilized in the synthesis of phospholipids." Baer & McArthur (107) have published details of a method for preparation of phosphorylcholine. Luecke & Pearson (108) and Entenman and co-workers (109, 110) have published methods for the determination of choline in phospholipids and tissues.

Taurog, Entenman & Chaikoff (110a) have presented data on the molal ratios of choline to phosphorus in plasma phospholipids of sixteen normal human subjects and of five dogs. It was assumed that in choline phospholipids there would be one molecule of choline for each atom of phosphorus. Practically all of the plasma phospholipids seemed to be of the choline containing type. Only five per cent or less of the phospholipids of human and canine plasma were found not to contain choline. The authors have compared their data with those of previous investigators.

In the last year the exact nature of the lipotropic agents which prevent deposition of fat in the liver has not been greatly clarified. The observations of Abels *et al.* (90), and of Entenman and co-workers (95, 96, 97) have been cited earlier in this review. Goodell, Hanson & Hawkins (111) have briefly summarized the literature showing that the liver is more resistant to such toxic agents as chloroform and arsphenamine after a high rather than after a low protein diet. They subjected dogs to a preliminary fast of one week and a low protein diet for two to fourteen and one half weeks. Then they tested the efficacy of methionine as a protective agent for the liver after intravenous injections of mapharsen. The dogs which were protected by methionine did not develop liver damage. The icteric index was used as a measure of liver damage and in some animals post mortum histological studies were made on the livers.

These authors emphasized that in order to protect the liver, adequate protein stores and good nutritional state should be developed in patients who are to be given arsenicals. György (88) suggested that there must be the right balance between methionine, cystine, and choline in the diet. Whether certain forms of protein such as that in milk are of more therapeutic benefit than the proteins of meat (112), cannot be decided until more is known about lipotropic agents in various types of fatty livers. In connection with the lipotropic action of amino acids, Horning & Eckstein (113) found in adult rats as much lipotropic effect from methionine and cystine as from cystine and enough casein to supply the same quantity of methionine. This is at variance with the results of Beveridge, Lucas & O'Grady (114), but the difference between the findings of these two groups of investigators may depend on the fact that the basal diet used by Beveridge and others contained 20 per cent protein in the form of gelatin and the basal diet used by Horning & Eckstein contained 5 per cent casein. Treadwell, Tidwell & Gast (115) have emphasized that the amount of methionine having a lipotropic action may be limited by the amount required for growth. The age of the rats and differences in the basal diets apparently lead to contradictory results. Roberts & Eckstein (116) have presented evidence to show that dimethyl sulfide, dimethyl disulfide, S-methylisothiurea, and methylxanthogenate have lipotropic effects, but that methionine sulfone given intraperitoneally or orally has no lipotropic effect. Miller (117) working with dogs, and Albanese, Holt and co-workers (118, 119), who studied nitrogen balance in two healthy males, have demonstrated the importance of methionine and cystine in the maintenance of nitrogen balance and conservation of tissue nitrogen.

*Kidney.*—Kidney lesions as well as fatty livers develop in rats on a choline-deficient diet. Patterson, Keevil & McHenry (100) have confirmed their earlier observations that choline deficiency diminished the phospholipid in the livers and kidneys of young rats. By the use of radioactive phosphorus they have demonstrated that this deficiency decreased the phospholipid turnover in the kidneys to a greater per cent than in the liver. In later papers (120, 121) these authors gave data showing that after young rats received a choline deficient diet for ten days the kidney weight, moisture, total lipid, and nitrogen increased, although the total phospholipid and choline decreased. Luecke & Pearson (108) found that after choline deficiency in four mature rats the free choline but not the total choline diminished per gram of rat kidney tissue. Since the review of Griffith (122) further compari-

sons of the effects of various lipotropic agents on liver and kidney tissue have been made (81, 91, 99).

Although hyperlipemia in nephrosis and in the nephrotic stages of glomerulonephritis has been recognized for many years little has been published on this subject recently even in articles on lipid nephrosis (123). Peters & Man (124) measured the serum lipids on one hundred and forty-two occasions in fifty-four patients with glomerulonephritis. Hypercholesterolemia occurred in some patients who had signs and symptoms associated with nephrosis, but who could not be placed in the categories of nephrosis or the nephrotic phase of glomerulonephritis. In the few (nineteen) observations on the ratio of free to total cholesterol the ratio usually was within the normal limits. The hypercholesterolemia was not consistently related to any single phenomenon of the disease. It was not inversely related to the level of serum albumin.

The hyperlipemia showed a different relation between the various serum lipid fractions than hyperlipemias observed in myxedema and obstructive jaundice. In glomerulonephritis, free fat was more intensely and consistently increased than in myxedema. The normal ratio of free to total cholesterol in renal disease is at variance with the elevation of free to total cholesterol in liver disease. The cholesterol: lipid phosphorus ratio in glomerulonephritis was usually normal although in obstructive jaundice this ratio was distorted due to a relatively greater increase in serum lipid phosphorus than in serum cholesterol.

After bilateral nephrectomy or ureteral ligation in dogs or monkeys, serum cholesterol, lipid phosphorus and total fatty acids rose (125). In the monkey the neutral fat also increased. Farr, Smadel & Holden (126) found a marked increase in both total lipid and cholesterol carbon in the plasma of rats sacrificed four to eighteen days after daily injections of kidney antiserum. The mechanism which caused the hyperlipemia in human glomerulonephritis, in nephrotoxic rats and in dogs and monkeys after bilateral nephrectomy or ligation of the ureters is not understood.

*Vitamins and enzymes.*—The recent investigations of Boxer & Stetten (127) do not agree with the observations enumerated by McHenry & Patterson, and others (91) that thiamine plays an essential role in the conversion of carbohydrate to fat. By combining the techniques of paired feeding and of isotopic analyses they demonstrated that loss of fat in thiamine-deficient animals resulted from diminished appetite and food intake. Three groups of five rats each were em-

ployed. Group A received the complete basal diet *ad lib.*; group B the same diet but without thiamine; and group C was limited to food intake of group B, but with thiamine present. After sixteen days each rat was injected with nearly pure deuterium oxide and given drinking water containing 6 per cent deuterium oxide. After five days rats were killed. The livers from each group were pooled, frozen with carbon dioxide, and used for subsequent analysis. Rats receiving the thiamine-deficient diet (group B) lost appetite and weight rapidly. Group C suffered similar weight loss although thiamine was present. The authors calculated the quantity of newly synthesized fatty acids in the livers. Normal rats (group A) had synthesized 2.25 gm. of fatty acids in contrast to 0.18 gm. by group B, and 0.27 gm. by group C. Furthermore, the saturated fatty acids were richer in deuterium than the singly unsaturated acids in the three groups of animals.

No evidence in favor of the "vitamin sparing action of fat" was found in a series of dietary experiments on six women conducted by Reingold, Nicholson & Elsom (128). After a preliminary observation period on normal diet each subject received in varying sequence for periods of ten to fifteen days a basal diet, a diet high in fat, and one high in carbohydrate. The caloric and thiamine (twice the normal requirement) intakes were kept constant. Bodily activity was constant and restricted. Thiamine in the urine, feces, and food was determined by the thiochrome method. Urinary excretion of thiamine was decreased in five of six women when the carbohydrate to fat ratio was increased. The high fat diet had the same effect as the basal diet on urinary thiamine. Excretion of thiamine in feces was not altered by changes in diet. Baldwin & Longenecker (129) found no differences in the fatty acids of tissues of normal and scorbutic guinea pigs. Experiments on the effects of iron and ascorbic acid on respiration of tissue suspensions have led Elliott & Libet (130) to suggest that ascorbic acid and iron may play a part in lipid metabolism. According to Thompson & Steenbock (131) the induction period for the oxidation of plant and animal fats was not changed by additions of  $\alpha$ -tocopherol unless antioxidants were removed by chromatographic adsorption.

Dam (132) found that the feeding of vitamin E to chicks produced increases in muscle cholesterol, provided 2 per cent of the basal diet was cholesterol. He also observed that vitamin E protected chicks from the encephalomalacia which results from the addition of the unsaturated fatty acid fractions of liver to basal diet (133). Lundberg and associates (134) raised weaned rats on vitamin E-free diets and then

analyzed their adipose tissues for tocopherol. After feeding a 50 mg. dose of tocopherol to vitamin E-deficient rats maximum deposition was not achieved until seven to ten days had elapsed. Larger doses resulted in increasing deposition. The amounts deposited indicated that fat depots may be major sites for storage of vitamin E. McHenry & Cornett (135) have concluded from a review of the literature that while there is experimental evidence that several of the B vitamins are necessary for the *in vitro* synthesis of fats from carbohydrates or proteins, there is only indirect and controversial evidence that vitamins may play a primary role in the metabolism of fats in animals.

Edwards (136) has presented evidence from experiments with cocarboxylase in depancreatized dogs which suggests that the course of conversion of fat to carbohydrate proceeds from acetoacetic acid to citric acid and thence to glucose. In a preliminary note Lehninger (137) has suggested that adenosinetriphosphate is required to initiate fatty acid oxidation.

#### MISCELLANEOUS

*Absorption and distribution.*—Reinhardt, Fishler & Chaikoff (55) observed the transfer of phospholipids labeled with radioactive P from plasma to thoracic duct and concluded that capillary and sinusoid walls are permeable to phospholipid molecules. Frazer's work (65, 66, 67) has been discussed in the section on intake and synthesis of lipids. He has concluded that his data can be explained by the partition hypothesis of fat absorption.

*Protein-lipid relationships.*—Chargaff & Bendich (138) studied the disintegration of thromboplastic protein as a result of freezing in the presence of ether. Partial removal of lipids from the apparently homogeneous complex produced a striking reduction in thromboplastic activity but left phosphatase activity. A number of papers on lipoproteins have appeared in foreign periodicals (139 to 144). A review of this subject by Schulman (145) appeared recently. Tayeau (146) had previously suggested that cholesterol in serum is linked with proteins as a consequence of similarity between ring structures. As contributing evidence he has reported that in cases of retention jaundice nearly all of the cholesterol and phospholipids of serum were extracted by ether. Serum of normal controls yielded no cholesterol.

The hypothesis long advocated by Bloor that the most active tissues contain the most phospholipids, was again supported by Kaucher *et al.* (105). Detailed analyses were made of beef brain, liver, heart, kidney,



lung, thymus, and intestine, and of muscle tissue of frog, turtle, veal calf, lamb, salmon, codfish, shrimp, beef, pig, and chicken. Finally, the eggs of the chicken and turtle were studied. Composite pooled samples of tissues were used for analysis by oxidative techniques previously described by the authors. The fractions considered were phospholipids, cephalin, lecithin and sphingomyelin, free and combined cholesterol, cerebroside, and neutral fat. The significance of this impressive body of data is limited by the fact that the types of physiological activity were vaguely defined and not quantitative.

After a reinvestigation of the dynamic effects of beef proteins, corn sugar, and lard in rats, Forbes & Swift (147) concluded that the prevalent idea that the dynamic effects of diets vary with their protein content is incorrect.

*Fat metabolism in various clinical conditions.*—Luzzatti (148) has studied serum lipids in the celiac syndrome. Bayles & Riddell (149) have compared plasma lipids in patients with rheumatoid arthritis, in similar patients receiving gold salt therapy, and in patients during pregnancy. Lepard (150) has reported a case of lipemia retinalis in a nondiabetic patient. In one hundred patients who had been subjected to an abnormally prolonged subsistence on a diet rich in cholesterol Shaffer (151) did not find any increase in incidence of coronary arteriosclerosis. Hirsch & Weinhouse (152) have reviewed the role of lipids in atherosclerosis and have concluded that the lipid deposits "develop in adult life without an appreciable elevation of the blood cholesterol or obvious abnormality of the blood lipids." Leary (153) by histological examinations has studied cholesterol in atheroma.

Fat and cholesterol content of fish sperm has been investigated anew by Schmidt-Nielsen & Sundsvold (154). Analyses of the lipid content of the respiratory tract fluid have been reported by Boyd *et al.* (155). The lipids of gonococcus have been studied by Boor & Miller (156).

*Red cell destruction and blood clotting.*—Considerable evidence has accumulated which indicates that lipids and closely related substances play a role in red cell destruction and in the clotting mechanism. Gruning (157) has found that ether or chloroform extraction of serum albumin reduces its antithrombic qualities to a minimum. Roy & Biswas (158) have studied the effects of cholesterol in controlling hemolysis of sheep cells by saponin, sodium oleate, cobra venom, vibrio, bacterial hemolysins, and bile salts. Johnson and co-workers (159) have summarized their work and that of others on the effects of serum lipids on erythrocytes. Lipemic serum increased the sus-

ceptibility of red cells to hypotonic hemolysis. Cells of pernicious anemia patients when mixed with lipemic serum were much more susceptible to hemolysis than the cells of normals. They have concluded that one function of the thoracic duct lies in preventing rapid entry of lipids into the blood stream, thereby decreasing the destruction of erythrocytes. Lee (160) has reported *in vitro* experiments on the effects of lecithin and cholesterol on normal and hemolytic serum.

*Methods.*—Various modifications of the Liebermann-Buchard colorimetric method and of other methods for the determination of cholesterol in tissues or blood have appeared recently (161 to 166). Alkalay (167) has presented evidence to support the theory that as human serum stood *in vitro*, the amount of esterified cholesterol increased. Methods for the determination of volatile or of unsaturated fatty acids in blood have been developed by McClendon (168), McAnally (169), and Marenzi & Cardini (170). MacLachlan (71) has published a method for the determination of the iodine number of whole phospholipids. Cardini & Serantes (171) have investigated procedures for extracting sphingomyelin from tissues.

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# THE METABOLISM OF PROTEINS AND AMINO ACIDS

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## GENERAL ASPECTS OF PROTEIN METABOLISM

*Protein synthesis.*—The mechanisms of intracellular protein synthesis continue to remain obscure. The best demonstration to date of enzymatic peptide-bond synthesis is that reported by Bergmann and co-workers and reviewed by Bergmann & Fruton (1). Whether this mechanism, that is, peptide-bond synthesis by proteolytic enzymes, actually represents the chief intracellular pathway for protein synthesis is uncertain. The position of thermodynamic equilibrium of this type of reaction is almost completely in favor of hydrolysis and speaks against this mechanism unless one invokes special conditions which would allow the reaction to proceed toward synthesis; that is, removal of synthetic products from the reaction medium due to insolubility, change in ionic or molecular activity, more nearly anhydrous conditions, etc. On the other hand, the proteolytic enzymes would seem to provide the factor of specificity which may be required for the synthesis of specific proteins. While reasoning by analogy is admittedly dangerous, it is nevertheless of more than passing interest to note that with our increasing knowledge of intermediary metabolism such synthetic reactions as those of urea, glycogen and starch, sucrose, etc., have been shown to be catalyzed by enzyme systems totally unrelated to the simpler and better known corresponding hydrolytic enzymes. Since these syntheses are known to be coupled ultimately with oxidation-reduction systems, the energy from which is transferred through intermediary phosphorylation reactions, it seems more than likely that peptide-bond synthesis is also coupled through energy rich phosphorylated intermediates with exergonic oxidation-reduction systems. It would appear that possible enzymatic systems coupling peptide-bond synthesis with high bond energy compounds of the phosphorylated type need to be investigated. The possibility exists that amino acids are phosphorylated on either the carboxyl or amino groups before condensation occurs. It is further possible that amino acids are not coupled as such but rather as combinations of phosphorylated  $\alpha$ -ketoacids and amino acids which give rise to ketopeptides [see Linderstrøm-Lang



(2)]. The possible pathways for the metabolic conversion of these keto-peptides to true peptides have been outlined by Herbst & Shemin (3). The experimental difficulties involved in this type of investigation are unquestionably great. On the other hand, the need and promise of such studies are even greater.

*Plasma and tissue proteins.*—The effect of intravenous administration of serum proteins, gelatin, protein digests, and amino acid mixtures on serum protein regeneration and nitrogen balance has been studied rather extensively during the past year. On the whole, the experimental findings tend to support the concept that plasma proteins are in dynamic equilibrium with tissue proteins (4, 5). Thus the injection of serum albumin to humans (6 to 9), plasma or serum protein to dogs (10), or plasma protein labeled with  $N^{15}$  to dogs (11) resulted in a relatively rapid loss of injected protein from the blood stream with a relatively late appearance of this nitrogen in the urine. This indicates that the proteins rapidly pass into the tissues where they are incorporated with tissue protein or slowly metabolized.

Gelatin as a substitute for plasma proteins has been shown to have a limited ability to contribute to the synthesis of plasma protein and hemoglobin in dogs, and may actually be toxic if administered for longer than three to seven days (12). Following intravenous injection of gelatin as much as 80 per cent can be recovered from the urine unchanged after forty-eight hours (13). Some evidence for the limited catabolism of gelatin after intravenous injection in dogs and man has been published by Brunschwig *et al.* (14).

The effectiveness of plasma or amino acids (synthetic mixtures or protein digests) in maintaining nitrogen balance and producing plasma proteins and hemoglobin has been reviewed by Whipple & Madden (15) and Elman (16). The comparative effect of amino acid mixtures and casein digests in producing plasma protein in depleted dogs is reported by Madden *et al.* (17). The ten essential amino acids plus glycine, given parenterally over a long period of time, were found to maintain nitrogen balance and produce as much new plasma protein as good dietary protein. No toxicity of the *D*-amino acids present in the synthetic *DL*-amino acids was noted.

The omission of histidine from a synthetic mixture of the other essential amino acids given intravenously to dogs did not adversely affect nitrogen balance until after three days (18). Bassett *et al.* (19) have administered parenterally a mixture of the ten essential amino acids to a human daily over a period of about one month. Nitrogen

balance was maintained with some evidence of serum protein regeneration. However, the patient was receiving varying amounts of protein orally during most of this time and consequently the effectiveness of the parenterally administered amino acids is difficult to evaluate.

A comparison of the effectiveness of enzymatic hydrolysates of casein, lactalbumin, and beef serum protein on serum albumin synthesis in hypoproteinemic dogs has been studied by Cox & Mueller (20). These digests, when fed to rats, were found to support growth about as well as the intact proteins. When given intravenously to hypoproteinemic dogs the three digests were found to be equally effective in regenerating plasma albumin. The casein and lactalbumin digests proved to have about the same potency values orally as intravenously. However, beef serum protein gave a potency value twice as great when given orally. The authors ascribe the latter finding to experimental difficulties. From this study the authors suggest that the differences previously reported by Weech (21) as to the ability of these proteins to regenerate serum albumin when fed unhydrolyzed to hypoproteinemic dogs may be due to differences in the rate of gastrointestinal proteolysis. While Cox & Mueller emphasize the fact that the potency values found by them for hydrolyzed casein given intravenously or orally were of the same order of magnitude as those found by Weech for unhydrolyzed casein given orally, they fail to point out that the potency values for lactalbumin and beef serum protein digests were only one-half to one-third those found by Weech for the intact proteins. The findings of Cox & Mueller, therefore, could be interpreted to indicate that when proteins of high potency value, such as lactalbumin and beef serum protein, are hydrolyzed and given intravenously they are only one-half to one-third as effective as when the intact proteins are fed orally.

The use of hypoproteinemic rats for assaying the serum protein regenerating ability of different proteins in the diet was reported by Cannon *et al.* (22). Of interest was the finding that bovine serum gamma globulin was far superior to bovine serum albumin. The inadequacy of human plasma proteins for rat growth has been demonstrated by Hegsted *et al.* (23). A low content of isoleucine in the human plasma proteins appeared to be responsible for the deficiency since supplementation with 0.5 per cent *dl*-isoleucine resulted in a near maximum response. The separation, concentration, and study of the properties of a group specific isohemagglutinin from human plasma was reported by Pillemer *et al.* (24). Edsall *et al.* (25) have investi-

gated certain of the proteins concerned in the blood coagulation mechanism.

Bálint & Bálint (26) determined the tyrosine, tryptophane, cystine, arginine, and histidine content of different plasma fractions, and of globin in hemoglobin, from cases of different types of anemias. No variations from normal were found. In a further paper (27) these authors analyzed proteins from ascitic fluid for the above amino acids, and found the values to be identical with those found in the corresponding plasma protein fractions. Louis & Lewis (28) analyzed the proteins from liver, muscle, and kidney of rabbits for total nitrogen and sulfur, and for the amino acids cystine, tyrosine, and tryptophane. No essential differences were found among well-fed animals, fasted animals, and animals poisoned with hydrazine and yellow phosphorus.

Feeding mice large amounts of tyrosine had no effect on the tyrosine content of tissue proteins (29).

*Amino acid requirements.*—The amino acid requirements of the chick have been reviewed recently by Almquist (30). Further data have been published by Hegsted (31) and Almquist & Grau (32) which indicate that leucine, threonine, phenylalanine, isoleucine, valine, and histidine are essential for maintaining weight. Glycine and glutamic acid are apparently required for growth, but not for maintenance of weight.

Kinsey & Grant (33) found that rats grew as well on a synthetic diet containing the ten essential amino acids as on a diet containing an equivalent amount of nitrogen in the form of casein. No evidence of toxicity due to the unnatural amino acids in the diet was noted, in contrast to the report of Albanese & Irby (34) [see also (18)]. Martin (35), who likewise noted no evidence of such toxicity, found that when casein, a casein digest, or a mixture of the ten essential amino acids was fed to rats, the relative growth values decreased in the order given. Of considerable interest was the finding that when succinyl-sulfathiazole was added to the above diets, the animals receiving the mixture of the ten essential amino acids all lost weight and died at the end of seven weeks. Since the diet contained all the known vitamins, it was suggested by Martin that the intestinal bacteria must synthesize amino acids essential to the host which are not present in the mixture of the ten known essential amino acids. The failure to realize optimum growth with gelatin supplemented with the essential amino acids in which gelatin is lacking was reported by Hier *et al.* (36). It was found that when diets contained 6 per cent glycine, 5 per cent *dl*-phenylala-

nine, or 5 per cent proline growth was inhibited. The authors suggest that the failure to obtain optimum growth with supplemented gelatin diets may be related to the toxic effect of these amino acids.

The effect of cystine, methionine, and histidine deficiency in the human was studied by Albanese *et al.* (37, 38). When the diet was deficient in methionine plus cystine, negative nitrogen balance ensued. Supplementation with methionine alone restored nitrogen equilibrium, suggesting that cystine is not required when methionine is present in the diet. Humans on a histidine-deficient diet remained in nitrogen equilibrium but lost weight. This confirms the finding of Rose *et al.* (39).

A complete survey of the growth requirements and metabolism of amino acids by microorganisms is beyond the scope of this review. However, information of considerable interest has been obtained particularly from studies on the use of lactic acid bacteria for microanalysis of amino acids. The successful use of *Lactobacillus arabinosus* 17-5 for analysis of valine and arginine (40), tryptophane (41), glutamic acid (42), leucine, valine, and arginine (43), and valine and leucine (44) in protein hydrolysates has been reported. The activity of the *d*-forms requires more detailed investigation. Dunn *et al.* (42), and Hegsted & Wardwell (45) found that *d*-glutamic acid and *d*-leucine were active. On the other hand, Stokes & Gunness (46) observed no activity with a series of *d*, *dl* and allo forms. Fox *et al.* (47) reported an inhibition of *Lactobacillus arabinosus* 17-5 by *d*-leucine in relatively high concentrations. Antibiotics such as gramicidin and tyrothricin, which have a high content of *d*-peptides, were found to inhibit in much lower concentrations. This effect has been suggested as a possible basis for the activity of these antibiotics by Fox *et al.* The observation by Schweigert *et al.* (44) that three different samples of *dl*-leucine had from 33 to 41 per cent activity rather than the theoretical 50 per cent also suggests an inhibition by *d*-leucine.

The use of a "leucineless" mutant strain of *Neurospora crassa* (48) for the determination of leucine in protein hydrolysates has been reported by Ryan & Brand (49). Glutathione has been shown to be an essential growth factor for certain strains of *Neisseria gonorrhoeae* (50).

#### INTERMEDIARY METABOLISM

*General.*—Shemin & Rittenberg (51) reported an excellent study on the metabolic interrelationships of various nitrogen compounds.

Glycine containing  $N^{15}$  was fed to rats for three days, after which the animals were sacrificed at intervals of naught, two, four, and seven days. Nonprotein nitrogen and proteins of various organs, amino acids of these proteins, total nitrogen, urea, and ammonia of urine were determined or isolated. The isotopic concentration was estimated in all of the above fractions. The rate of disappearance or appearance of  $N^{15}$  in the different fractions determined or isolated in the successive intervals served as a measure of the relative metabolic activity. Among the many findings and metabolic correlations established, the following seemed of particular interest: In seven days, one half of the liver protein was replaced by nitrogen from the diet and other proteins; urea and the amidine group of arginine from liver proteins had approximately the same concentration of  $N^{15}$ , thus providing further evidence in support of the Krebs-Henseleit urea cycle; the isotope concentration of the amidine group of carcass arginine was much lower than that of urinary urea, which supports the view that the liver is the chief site of urea formation; different muscle proteins varied considerably in their metabolic activity, some proteins were very reactive, others relatively inert; the nonprotein nitrogen fractions differed in their isotope concentration from organ to organ; in the synthesis of ornithine in the rat, dietary nitrogen was incorporated in both the  $\alpha$ - and  $\delta$ -amino groups. A group of rats bearing transplanted sarcoma R-39 was also studied. It was found that the total protein of the tumor incorporated  $N^{15}$  about as rapidly as liver but its rate of release was only about one half that of liver.

Shemin & Rittenberg (52) could find no evidence of symmetrical synthesis of amino acids after feeding *dl*-glutamic acid, *dl*-tyrosine, and the latter along with ammonium citrate containing  $N^{15}$  to riboflavin-deficient rats.

*Deamination.*—Blanchard *et al.* (53) have investigated in detail the properties of an *l*-amino acid oxidase prepared from rat kidney and liver. The enzyme was found to catalyze the oxidation of all naturally occurring mono-amino-mono-carboxylic acids, except glycine, threonine, and serine, to ammonia and the corresponding  $\alpha$ -keto acid. Glutamic acid, aspartic acid, lysine, ornithine, and arginine were not attacked. Proline was oxidized with splitting of the pyrrolidine ring to form  $\alpha$ -amino  $\delta$ -ketovaleric acid. Purification of this enzyme and some of its properties are reported in a later paper (54). While the enzyme is a flavoprotein, the prosthetic group is not flavin adenine dinucleotide. Stumpf & Green (54a) have studied the *l*-amino acid oxidase

systems in *Proteus vulgaris*. A relatively stable system associated with the insoluble particles was prepared by ultrasonic disintegration.

The oxidation of *l*-amino acids using tissue slices has been studied by Edlbacher & Grauer (55). Evidence for the oxidation of *l*-alanine and *l*-aspartic acid by different enzyme systems was reported on the basis of different inhibitor effects. The oxidation of both amino acids was found to be inhibited by cyanide. It is of interest to note that while the *l*-amino acid oxidase of Blanchard *et al.* (53) oxidized *l*-alanine, but not *l*-aspartic acid, it was not sensitive to cyanide. Edlbacher & Grauer reported the disappearance of ammonia when guinea pig kidney slices were incubated with lactate or succinate plus *l*-alanine or ammonia. Apparently they have rediscovered the glutamine synthesis reaction previously reported by Krebs (56). Karrer & Appenzeller (57) studied the specificity of *d*-amino acid oxidase (pig kidney powder) using a variety of methyl, ethyl, butyl, acetyl, and dimethyl derivatives of *dl*-alanine. A comparison of the activity of *d*-amino acid oxidase from livers of trout, pigeon, seagull, and hen revealed marked species differences in regards to substrate specificity. Horowitz (58) has found that *Neurospora crassa* contains a *d*-amino acid oxidase which is similar in most of its properties to that found in mammalian tissues.

Ratner *et al.* (59) have prepared a purified flavoprotein called glycine oxidase from liver and kidney of several species. The enzyme catalyzes the oxidation of glycine to glyoxylic acid plus ammonia, and the oxidation of sarcosine to glyoxylic acid plus methylamine. The prosthetic group is flavin adenine dinucleotide.

*Transamination.*—The rate of transamination in oat seedlings and in different species of bacteria has been investigated (60, 61). In contrast to previous reports, the rate of transamination in bacteria and plants is considerably higher than that observed in most animal tissues. The relative  $Q_{TN}$  values of *B. coli*, animal, and plant tissues are shown in Table I. A possible relationship between transamination and protein synthesis is suggested from these studies. Transamination in model and in enzyme-catalyzed systems has been reviewed recently by Herbst (62).

*Decarboxylation.*—A high degree of purification of the *l*-lysine and *l*-tyrosine decarboxylase systems of *Escherichia coli* and *Streptococcus fecalis* was reported in a series of papers by Gale & Epps (63, 64, 65). The amino acid decarboxylases have been shown to have a co-factor in common which is dissociable from the apoenzyme. The co-factor is

widely distributed in animal tissue, plant tissue, yeasts, and bacteria. The compound, which is different from any known co-factor, has been obtained in practically pure form and contains carbon, hydrogen, and nitrogen, but no phosphorus or sulfur. The absence of phosphorus and other properties make it appear that the co-factor isolated by the English investigators is not identical with pyridoxal phosphate which

TABLE I

$Q_{TN}^{\dagger}$  VALUES OF *B. coli*, ANIMAL, AND PLANT TISSUES\*

Tissue	$Q_{TN}$
<i>Bacillus coli</i> .....	3,900
Oat seedlings (96 hrs.) .....	5,650
Brain (rat) .....	2,800
Liver (rat) .....	2,200
Kidney (rat) .....	1,750
Heart muscle (rat) .....	3,330
Purified transaminase (from beef heart muscle) .....	10,300

\* From Lichstein & Cohen (61).

† Substrates: *l*-glutamic acid plus oxalacetic acid.  $Q_{TN} = \frac{\mu\text{l. aspartic acid formed}}{\text{mg. N} \times \text{hrs.}}$ .

Gunsalus *et al.* (66) report to be the coenzyme for *l*-tyrosine decarboxylase. The purified *l*-tyrosine decarboxylase system from *Streptococcus fecalis* acts on both *l*-tyrosine and *l*-3,4-dihydroxyphenylalanine (dopa) in contrast to animal tissues which have been reported to have separate enzymes for the decarboxylation of these compounds (67).

#### INDIVIDUAL AMINO ACIDS

*Glutamic acid, proline, and hydroxyproline.*—The conversion of *d*-glutamic acid to *d*-pyrrolidonecarboxylic acid has been demonstrated by feeding rats *dl*-glutamic acid containing  $N^{15}$  in the amino group and deuterium attached to the  $\alpha$ - and  $\beta$ -carbon atoms (68). As much as 73 per cent of the ingested *d*-form was found to be excreted in a form in which the amino group was not free and which contained the same deuterium concentration as that originally present in the *dl*-glutamic acid. This indicated that no reaction involving the  $\alpha$ - and  $\beta$ -hydrogen atoms had taken place. In a second experiment, *d*-pyrrolidonecarboxylic acid was actually isolated from the urine in approximately 40 per cent yield after feeding *dl*-glutamic acid.

Pedersen & Lewis (69) studied the excretory "extra" nitrogen after feeding rabbits *l*-glutamic acid, pyrrolidonecarboxylic acid, *l*-proline, and *l*-hydroxyproline. *l*-Glutamic acid was found to be readily utilized as evidenced by the fact that the "extra" nitrogen of the urine

appeared chiefly as urea. In contrast, the feeding of pyrrolidonecarboxylic acid resulted in a much smaller excretion of urea and a considerable portion, as much as 30 per cent, as undetermined nitrogen. The amino nitrogen excretion was not increased. The feeding of *l*-proline resulted in a considerable increase in "extra" urea nitrogen with about a 10 per cent increase in amino nitrogen. Feeding hydroxyproline gave rise to practically no "extra" urea nitrogen and resulted in an amino nitrogen increase of as much as 38 per cent of the nitrogen fed. These experiments indicate that the metabolic pathways *in vivo* of proline and hydroxyproline are not identical, and further, it would seem, that hydroxyproline is not converted to proline.

Additional information on the metabolic relationships of proline, hydroxyproline, and glutamic acid was provided by the experiments of Stetten & Schoenheimer (70). These workers synthesized *l*-proline with deuterium on the carbon skeleton and  $N^{15}$  in the amino group. When this was fed to rats, 40 per cent of the isotopic nitrogen appeared in the urine as urea and ammonia. The remainder was distributed in the protein and nonprotein nitrogen fractions of the different tissues. Isotopic analysis of the proline isolated from the tissue proteins indicated that at least 30 per cent of the proline of the proteins from internal organs and 7 per cent from the carcass proteins was replaced by dietary proline in three days. Glutamic acid isolated from the tissues was found to contain both deuterium and  $N^{15}$ , thus establishing the *in vivo* oxidation of proline to glutamic acid. The metabolic conversion of proline to ornithine was demonstrated by the finding of deuterium in the ornithine isolated from tissue arginine.  $N^{15}$  was found not only in the amidine group of the latter but also in the  $\alpha$ - and  $\delta$ -amino groups of the former. Isotopic analysis of hydroxyproline isolated from carcass proteins indicated that about 25 per cent of this amino acid was formed in three days from fed proline. This study would seem to establish unequivocally the *in vivo* conversion of proline to glutamic acid through the intermediates hydroxyproline and ornithine.

The metabolism of the diketopiperazines of *l*-proline and *l*-hydroxyproline in the rabbit has been studied by Schlütz (71). On the basis of recovery from urine and feces, about 50 per cent of both anhydrides were found to be broken down when injected subcutaneously. When given orally the hydroxyproline anhydride was completely broken down while the proline anhydride was metabolized to the same extent as when given subcutaneously. Attempts to demonstrate splitting of



the diketopiperazines by tissue brei, slices, extracts, and by organ perfusion were unsuccessful.

*Histidine*.—The decarboxylation of *l*-histidine to histamine following parenteral administration to guinea pigs is reported by Holtz & Credner (72). The kidney is considered to be the chief site of this conversion by the authors. Holtz *et al.* (73) found that *d*-histidine given parenterally to guinea pigs is excreted practically completely and unchanged in the urine. In contrast, rats utilize about 50 per cent of injected *d*-histidine. These differences are thought to be due to the different concentrations of *d*-amino acid oxidase and histidine decarboxylase in the tissues of the two species.

Sakami & Wilson (74) found that *dl*-1-methyl histidine, while not toxic to young rats, failed to promote growth when added to a histidine-deficient diet. Since rat growth is known to be supported by either *d*- or *l*-histidine, the implication of this finding is that 1-methyl histidine cannot be demethylated. The synthesis of *dl*-1-methyl histidine was described in an accompanying paper (75).

Edlbacher & Grauer (76) described an enzyme system called *dl*-histidase which oxidized both *d*- and *l*-histidine. The enzyme was inhibited by cyanide but not by arsenious oxide. A co-factor of unknown nature, which was not alloxazine-adenine-dinucleotide, was required. The oxidation proceeded without rupture of the imidazole ring and with the formation of but little ammonia. The latter finding, however, may have been due to the conversion of ammonia to some other product during the course of the reaction.

Anrep *et al.* (77) found that carnivora excrete histamine chiefly in the conjugated form and that herbivora excrete histamine in the free form. Administration of meat, but not purified protein, leads to a considerable excretion of conjugated histamine. More than 60 per cent of the total histamine content of the mouse was found to be present in the skin (78).

*Lysine*.—The effect of a low lysine diet on young rats has been studied by Harris *et al.* (79). In addition to cessation of growth (but no weight loss) a decrease in serum protein concentration and histological changes were observed which were consistent with the effects of a general inhibition of protein formation. Neuberger & Sanger (80, 81) fed lysine-deficient rats  $\epsilon$ -N-acetyl-*l*-lysine and  $\alpha$ -N-acetyl-*l*-lysine,  $\epsilon$ -N-acetyl-*d*-lysine, and  $\epsilon$ -N-methyl-*dl*-lysine. Only the first and last of these compounds were found to be available for growth. A detailed study of the metabolism of *d*- and *l*-lysine and various derivatives has

been carried out by the same investigators (82). Both nitrogen atoms of *l*-lysine were found to be converted to urea after feeding to rats but *d*-lysine was excreted largely unchanged. *l*-Lysine did not disappear to any significant extent when incubated with slices of kidney, liver, brain, and heart. Kidney slices were found to be capable of demethylating  $\epsilon$ -N-methyl-*dl*-lysine oxidatively. This finding would explain the availability of this compound for growth when fed to lysine-deficient rats. *d*- and *dl*-Lysine and  $\epsilon$ -N-methyl-*dl*-lysine were not attacked by crude and purified *d*-amino acid oxidase preparations. However,  $\epsilon$ -N-acetyl- and  $\epsilon$ -N-benzoyl-*dl*-lysine were found to be oxidized at a moderate rate. From this it is suggested by the authors that the presence of the terminal basic amino group of lysine and methyl lysine prevents their oxidation by the *d*-amino-acid oxidase system. In discussing the intermediary metabolism of lysine, Neuberger & Sanger consider it a possibility that acetylation of the  $\epsilon$ -amino group may precede oxidation of the  $\alpha$ -amino group. [Unpublished experiments are referred to in which it was shown that while *l*-amino acid oxidase (53) does not oxidize *l*-lysine, it slowly but definitely oxidizes  $\epsilon$ -acetyl-*l*-lysine.] The resulting  $\alpha$ -keto acid could be deacetylated and decarboxylated to yield  $\delta$ -amino-valeric acid, or oxidized to glutaric acid. On the other hand, the possibility is considered that the first step in the metabolism of lysine is the oxidation of the terminal amino group to form  $\alpha$ -amino-adipic acid, which in turn could be oxidized to glutaric acid. Both mechanisms would explain many of the known metabolic peculiarities of lysine and are not in conflict with any known facts.

*Methionine and cystine*.—Earlier experiments have indicated that the conversion of methionine to cystine involves the following steps:

- a) demethylation of methionine to homocysteine (83),
- b) reaction of homocysteine with serine to form cystathionine (83, 84), and
- c) cleavage of cystathionine to form cysteine (85, 86).

Further evidence in support of the above scheme has recently been reported by du Vigneaud *et al.* (87) who fed methionine containing  $S^{34}$  and  $C^{13}$  in the  $\beta$ - and  $\gamma$ -positions. Cystine isolated from the hair of these animals was found to contain no significant amount of  $C^{13}$  but as much as 80 per cent  $S^{34}$ . This experiment proves that the carbon chain of methionine is not utilized in the conversion of methionine to cystine. Binkley (86) has found that the cleavage of cystathionine is catalyzed by an enzyme system from rat liver consisting of a liver

protein fraction, adenosinetriphosphate, and either magnesium or zinc ions. The indications are that the cleavage of the carbon-sulfur bond requires the high energy phosphate of adenosinetriphosphate and that the cleavage product other than cysteine is the phosphorylated hydroxyamino acid, phosphohomoserine. Lanthionine, the next lower thioether homologue of cystathionine, was also broken down by rat liver tissue to form cysteine.

The net effect of reactions (b) and (c) is the exchange of the sulfhydryl group of homocysteine with the hydroxyl group of serine. Binkley (86) has termed this transfer transsulfuration. A more euphonious and chemically more descriptive term, it seems to the writer, would be transthioation.

Normal human adults were found to excrete from 274 to 494 mg. of methionine daily (88). This represents about 6 per cent of the total urinary amino nitrogen.

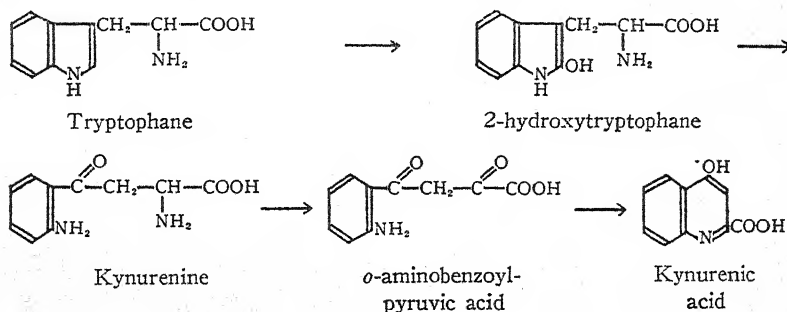
*Tryptophane*.—Albanese & Frankston (89) reported a difference in the metabolism of *dl*- and *l*-tryptophane. When the former was fed to humans it was observed that the urine contained a substance which when treated with 0.1 *N* iodine solution formed a heavy red-dish-purple precipitate. This substance upon isolation had properties characteristic of indigo red. This substance appears to be similar if not identical with that recently studied by Schales & Schales (90), who showed it to be present in many normal urines and in most pregnancy urines. Previous to these studies, Simola (91) reported the formation of a red pigment soluble in amyl alcohol following the addition of iodine solution to urines. The reaction was found to be negative in 10 per cent of the pregnancy urines and in about 60 per cent of the urines of normal and diseased patients. The amount of the substance recovered by Albanese & Frankston from the urine after feeding *dl*-tryptophane was large enough to account for the metabolism of most of the *d*-form. This fact indicates that the *d*-form is not available to the human. These authors point out that in human nutritional studies twice the quantity of *dl*-tryptophane is required to obtain the effect of the *l*-form.

Ried *et al.* (92) fed indole-3-pyruvic acid, tryptophane, indole-3-propionic acid, indole-3-lactic acid, indole-3-acetic acid, *N*-methyl-*l*-tryptophane, indole plus serine, and kynurenic acid to pyridoxine-deficient rats and found no excretion of xanthurenic acid (93) in the urine. However, kynurenine and *l*-tryptophane when fed gave rise to xanthurenic acid in the urine of pyridoxine-deficient rats. When xan-

thurenic acid was fed to rats which received pyridoxine, none of the acid could be found in the urine. The pyridoxine-deficient rat excreted this substance in the urine unchanged.

The synthesis of indole and of tryptophane by *Neurospora crassa* was reported to involve the intermediate formation of anthranilic acid, which was found to accumulate in the medium of a mutant strain (94). The synthesis of tryptophane by the same strain has been shown to occur by the direct interaction of indole and glycine (95). The formation of indole from tryptophane by *Escherichia coli* is a reversal of the latter reaction.

Butenandt *et al.* (96) have reinvestigated the chemical structure of *l*-kynurenine and have shown it to be *o*-aminophenacylaminoacetic acid. Proof of this structure was established by synthesis of *dl*-kynurenine. Comparison of the *l*-kynurenine with the synthetic *dl*-form as to eye pigment formation by *a-Ephestia* pupae revealed the racemic form to have one half the activity of the *l*-form, indicating that the *d*-form is not active as a chromogen. These authors further studied the V<sup>+</sup> hormone of Tatum & Haagen-Smit (97) who reported this hormone to be synthesized by certain bacteria. Analysis by Tatum & Haagen-Smit of the isolated crystalline material indicated it to be a sucrose half-ester of kynurenine. Butenandt *et al.* were led to reinvestigate this substance in view of the fact that the structure newly assigned to kynurenine includes but one carboxyl group. The latter investigators were able to show that the substance isolated by Tatum & Haagen-Smit is readily reproduced by crystallization of kynurenine from water in the presence of excess sucrose. Thus the so called V<sup>+</sup> hormone produced by certain bacteria is in fact kynurenine and its isolation as a sucrose half-ester is an artifact. On the basis of the new formula for kynurenine, its formation from tryptophane and its conversion to kynurenic acid is readily apparent from the following reactions:



*Tyrosine, phenylalanine, and dihydroxyphenylalanine (dopa).*—A comparative study of the aerobic metabolism of tyramine, *l*-tyrosine, and phenol by rat tissues (98) revealed that heart and skeletal muscle tissues are capable of both deaminating and breaking the ring of tyramine. Kidney and liver, it was found, could only deaminate the latter compound. Tyrosine, on the other hand, was found to have its ring broken by all these tissues with the exception of heart muscle. No significant deamination of tyrosine was observed with these tissues with the possible exception of liver.

Lan & Sealock (99) reported that liver slices from scorbutic guinea pigs are unable to oxidize *l*-tyrosine. The administration of ascorbic acid either *in vivo* or *in vitro* was found to restore the liver's capacity for tyrosine oxidation. The suggestion by these authors that ascorbic acid is a "necessary and key component of the enzyme systems responsible for the oxidation of the amino acid" is hardly warranted on the basis of the experiments reported. Similar effects of ascorbic acid added to liver slices from scorbutic guinea pigs have been reported by Quastel & Wheatley (100) for crotonic acid and butyric acid oxidation.

The production of a hydroxyphenyl compound, possibly tyrosine, from *l*-phenylalanine by guinea pig or rat liver slices has been observed by Bernheim & Bernheim (101).

Holtz, Credner & Strübing (102) reported that the hyperglycemia observed in rabbits after injection of dihydroxyphenylalanine (*dopa*) is not due to the dihydroxyphenylalanine but rather to its decarboxylation product, hydroxytyramine. Only the natural form of *dopa* is active in the rabbit. In a further study, Holtz & Credner (103) found that the conversion of *d*-dihydroxyphenylalanine to the *l*-form takes place in the rat, but not in the guinea pig or rabbit. The evidence for this is based on the fact that *dopa* decarboxylase is specific for the *l*-form. When *dl*-dihydroxyphenylalanine was fed to rats, more hydroxytyramine was excreted in the urine than was to be expected from an equivalent amount of the *l*-form alone. The higher *d*-amino acid oxidase activity of the rat tissues is considered by the authors to be responsible for the rat's ability to carry out this conversion.

*Thyroxine and diiodotyrosine.*—The synthesis of thyroxine and diiodotyrosine by rat thyroid gland has been shown to be depressed by thiouracil feeding (104). A similar effect is reported after feeding thiourea (105). The inhibitory effect of thiourea and related compounds, and of different sulfonamides on the synthesis of thyroxine

and diiodotyrosine from inorganic radioactive iodide by thyroid slices was studied by Franklin *et al.* (106, 107).

#### SPECIAL ASPECTS OF NITROGEN METABOLISM

*Acetylation of amino groups.*—Fishman & Cohn (108) investigated the acetylation of phenylaminobutyric acid (*d*- and *l*-forms), *p*-aminobenzoic acid and sulfanilamide in rats fed deuterium oxide. In all instances the excreted acetyl derivatives contained about the same concentration of deuterium in the acetyl group. On the basis of previous studies and the present studies, the authors point out that whatever the chemical nature of the acetylating agent, be it acetic acid or a related compound, it must be formed in the body by an irreversible reaction during which hydrogen from body water is introduced into the molecule. In a related study, Bloch & Rittenberg (109) fed *dl*-phenylaminobutyric acid to rats in addition to the following compounds: sodium dideuterio propionate, sodium dideuterio butyrate, sodium *n*-deuterio valerate, deuterio ethyl palmitate, 10, 11-dideuterio ethyl undecylate; deuterio *dl*-alanine, ethyl deuterio myristate, and ethyl deuterio stearate. The excreted *l*-acetyl phenylaminobutyric acid was isolated and the deuterium content determined. Of these compounds, propionic acid and 10, 11-dideuterio undecylic acid failed to give rise to acetyl groups. Of considerable interest was the finding that *dl*-alanine was two thirds as active as acetic acid in yielding acetyl groups. This indicates that acetic acid is an intermediate in the catabolism of alanine or of pyruvic acid. In a continuation of this study, Bloch (110) found that feeding *dl*-deuterio leucine and deuterio isovaleric acid resulted in considerable acetylation of phenylaminobutyric acid. In contrast, feeding *dl*-deuterio valine and deuterio isobutyric acid resulted in no acetylation. From these results, Bloch concluded that isovaleric acid is an intermediate in the catabolism of leucine. Binkley, Wood & du Vigneaud (111) reported further studies on the *in vivo* inversion of *d*-amino acids to acetyl *l*-derivatives. The major parts of phenyl-*d*- and benzyl-*d*-cysteines, when fed to rats, were found to be converted to the corresponding N-acetyl-*l*-amino acids. Direct acetylation was found to occur also to a small extent. Of interest was the finding that direct acetylation of phenyl-*d*- and benzyl-*d*-cysteines was increased when the aromatic ring was substituted with a bromine atom. That this effect is not a general one, however, is seen from the fact that *p*-bromo-*d*-benzyl-homocysteine showed little or no direct acetylation. When the *l*-forms of the above amino acids, or N-acetylbenzyl-

*d*-cysteine, were fed, no inversion was observed. *p*-Fluorophenyl-*l*-cysteine was found to be excreted by the rat as *p*-fluorophenylmercapturic acid (112). The acetylation of primary aromatic amines by guinea pigs and by humans was studied by Zehender (113). *p*-Aminobenzoic acid, sulfathiazole, and sulfanilic acid were found to be excreted as acetyl derivatives. Aniline, in contrast to its ready *in vitro* acetylation, was not acetylated *in vivo*.

*Purines, pyrimidines, nucleic acids and nucleoproteins.*—Plentl & Schoenheimer (114) fed guanine, creatine, guanidoacetic acid, uracil, and thymine labeled with N<sup>15</sup> to rats and pigeons. Nucleic acids of the combined internal organs were then separated and hydrolyzed. Guanine, adenine, cytosine, and thymine were isolated and their isotopic nitrogen content determined. The nitrogen fractions of the urine were also analyzed. In contrast to all previous studies in which a variety of labeled metabolites were fed and later found to be incorporated in the tissue constituents, the purines and pyrimidines were not incorporated into the tissue nucleoproteins but rather were immediately metabolized to their respective end products. No metabolic relationship between the creatine cycle and the purines and pyrimidines was observed. Further, there was no evidence of the conversion of pyrimidines to purines. In the opinion of these authors, the nucleoproteins are synthesized either from molecules smaller than the purines and pyrimidines, or from larger molecules such as nucleosides, nucleotides, or nucleic acids.

The nucleic acid and nucleotide content of embryonic and adult tissue has been determined by Davidson & Waymouth (115). Embryonic tissue tends to have a higher nucleic acid content than the corresponding adult tissue. A study of the distribution of desoxyribonucleic and ribonucleic acids supports the view that the former is located in the nucleus and the latter chiefly in the cytoplasm. Acid-soluble purine nucleotides were found to be present in lower concentration in embryonic tissues. The metabolic turnover of nucleic acids in relation to growth has been investigated by Brues *et al.* (116) using radioactive phosphorus. It was found that the turnover of desoxyribonucleic acid phosphorus was very slow in contrast to most other cell constituents. During rapid growth, as in regeneration of liver, large amounts of radioactive phosphorus were taken up by desoxyribonucleic acid. Liver tumor, which had a growth rate between that of resting and regenerating tissue, showed an intermediate rate of radioactive phosphorus uptake. Kosterlitz (117) studied the effect of dietary protein

on the relative concentrations of protein, phospholipin, and nucleic acid fractions of liver cytoplasm. The relative concentrations of protein and phospholipin were found to remain remarkably constant when the protein content of the diet was varied. The nucleic acid fraction increased slightly with a decreasing protein intake due to the relative increase in nuclear material. These findings support the view that the changes in protein contents of livers observed after fasting and diets low or high in protein are due to changes in the cytoplasm content of the liver. About 25 per cent of rat liver cytoplasm appears to be labile to dietary variations.

*Allantoin.*—Interest in allantoin as a purine metabolite has led to the publication of three different studies on the content of this substance in blood. Young *et al.* (118) developed a colorimetric method for allantoin which, when applied to dog blood, gave values of from 0.8 to 2.3 mg. of allantoin per 100 ml. Blood from a pure Dalmatian dog averaged 0.4 mg. per 100 ml. This method, with some modification, was employed by Christman *et al.* (119) in a study of the allantoin content of bloods of different species. Since glucose, uric acid, and ergothionine were known to interfere, the glucose was removed by yeast treatment and corrections made for uric acid and ergothionine in each determination. Values ranging from 0.33 mg. per 100 ml. of blood for the hog to 2.61 for the cow were reported. The bloods of different species examined and found to fall within this range were those of the calf, sheep, rabbit, dog, rat, and horse. When the above corrections were applied to chicken and human bloods, negative values were obtained for allantoin. Of interest was the observation that the allantoin content of blood from black and white rabbits was about twice that found in blood from albino animals. A new colorimetric method for the determination of allantoin has been published by Archibald (120). Application of the method to normal human fasting plasma gave values of from 0.3 to 0.6 mg. of allantoin per 100 ml. It is possible that the negative values for human blood reported by Christman *et al.* were due to overcorrection for uric acid and ergothionine. While Archibald's method is not affected by these substances, no claim is made for its absolute specificity and thus his values may be too high.

The urinary excretion of allantoin following oral and intravenous administration to dog and man has been investigated by Young *et al.* (121). The ability of certain bacteria from the human intestinal tract to decompose allantoin has been demonstrated by Young & Hawkins (122).



*Creatine.*—The enzymatic synthesis of creatine from sarcosine plus ammonium carbonate by muscle brei is reported by Beard (123). The data presented appear hardly adequate to support this claim. Menne (124) has continued his previous studies on the origin of muscle creatine (125, 126). The earlier work by Menne indicated that arginine and histidine were precursors of creatine in frog skeletal muscle brei. In addition, glycoamine and choline were found to increase creatine synthesis. In his more recent work, Menne reports the synthesis of creatine from arginine by an enzyme system consisting of frog or rabbit muscle myosin plus an aqueous frog muscle *kochsaft* which is said to contain a co-factor. Frog muscle myogen or globulin X, as well as beef plasma albumin, globulin, or fibrinogen could not replace myosin in the system. While Menne reports no studies as to the nature of the co-factor, it is conceivable that the *kochsaft* contained sufficient sarcosine to react with the amidine group of arginine to form creatine. Confirmation of the synthesis of creatine from arginine using purified myosin would be highly desirable in view of the key role myosin is known to play in adenosinetriphosphate breakdown and muscle contraction. The fundamental importance of such a reaction is further seen from the fact that invertebrate muscle contains arginine phosphate rather than creatine phosphate.

The creatine content of skeletal muscle is not significantly lowered when a choline deficient diet is fed to the chick (127), the rat (128), and the dog (129).

Creatine and creatinine excretion in young normal adult males and females has been studied by Albanese & Wangerin (130). From this study it would appear that creatinuria is normal for the adult male, and further that the creatinine excretion of males and females, while uninfluenced by protein intake, may show a daily variation of as much as 25 per cent.

*Nitrogen compounds and fat metabolism.*—Choline deficiency results in a decrease in phospholipid turnover as well as in a decreased concentration of phospholipid in the liver and kidneys of rats (131, 132). The fatty liver and hemorrhagic kidneys which result from a choline deficiency are apparently due to this decrease in phospholipid turnover. Further evidence in support of the decreased rate of phospholipid turnover in choline deficiency is provided by the study of Boxer & Stetten (133) in which choline containing  $N^{15}$  was fed to both normal and choline deficient animals. Luecke & Pearson (134), using a microbiological method, have determined the free choline con-

tent of animal tissues. About 2 per cent of the total choline is present in the free form. Incorporation of 1 per cent choline in the diet of rats resulted in an increase of free choline in the kidney, but not in the liver and brain.

The metabolism of phosphorylcholine, containing radioactive phosphorus, in rats has been investigated by Riley (135). Following injection, phosphorylcholine was found to be rapidly broken down to yield inorganic phosphate. There was no evidence from this study that phosphorylcholine as such was utilized in the synthesis of phospholipid. The effect of pure amino acids and different fractions of a caseinogen digest on liver lipids has been studied by Channon *et al.* (136). Of fourteen pure amino acids tested, glutamic acid, tryptophane, and tyrosine were the only ones found to have some lipotropic activity. Some factor which affected liver fat deposition, other than cystine, methionine, or tyrosine, was reported to be present in a butanol-soluble amino acid fraction of a caseinogen digest. On the other hand, Horning & Eckstein (137) have presented evidence which indicates that the lipotropic action of casein in the diet of rats can be explained entirely on the basis of its methionine content. Beveridge *et al.* (138) found that there was no significant difference in the lipotropic activity of methionine when fed as the free amino acid or in casein, provided the level of the essential amino acids in the diets was the same. However, these authors stress that the lipotropic activity of methionine is markedly influenced by the level of other essential amino acids and that the possibility that other factors are present in casein which have lipotropic activity can not be excluded. Treadwell *et al.* (139) arrived at a similar conclusion from their studies. Their results indicate that not only is the amount of dietary methionine which is available for growth determined by the amounts of the other essential amino acids in the diet, but also that the availability of dietary methionine for lipotropic activity is limited by the amount used for growth. It is likely that differences in the observations reported by different workers in regard to the relative lipotropic effect of free methionine as compared with that of methionine in protein are real and are due to many factors, such as variations in age or size of animals, composition of diet, etc. This subject has been reviewed in some detail by McHenry & Patterson (140).

A protein fraction has been separated from pancreas by Entenman *et al.* (141) which is capable of maintaining a normal liver fat content of depancreatized dogs.

## MISCELLANEOUS

Studies on renal amino acid clearance in dogs have been carried out by Pitts (142, 143, 144) and Goettsch *et al.* (145). The absorption of individual amino acids and a casein hydrolysate from the gastrointestinal tract of chicks has been investigated by Kratzer (146). In general, the increase in blood amino acid levels after oral administration of amino acids was found to be similar to that observed in mammals. Forbes *et al.* (147) studied the associative dynamic effects of protein, carbohydrate and fat, and found that the dynamic effects of diets are not the additive effects of their components, and further, that these are not predictable from the protein content. In view of this, the authors feel that the dynamic effects of individual foodstuffs are without significance as constants. The effect of ingestion of large quantities of beef blood and muscle and of a protein digest on blood urea and amino acid levels, urinary urea excretion, and urea clearances was studied by Free & Leonards (148). It was observed that the normal gastrointestinal tract is able to handle large amounts of unhydrolyzed protein more readily than equivalent amounts of amino acids. The limiting factor which determined the maximum intake of protein or amino acids appeared to be the rate of intestinal absorption. Maximum urea clearance as well as urinary urea excretion were found to be increased following ingestion of these large quantities of proteins or amino acids.

Further evidence for an increased protein catabolism in the peripheral tissues of the rat in hemorrhagic shock is presented by Russell *et al.* (149) and Engel *et al.* (150). A similar finding is reported by Hoar & Haist (151) for dogs in traumatic shock. In both sets of experiments the observed persistent rise in blood amino nitrogen levels was explainable on the basis of an alteration of liver function during the shock state of the animals.

The effect of amino acids on the multiplication of bacteriophage has been studied by Spizizen (152). It is reported that glycine, glycine anhydride, and hippuric acid are capable of supporting phage multiplication without concomitant bacterial growth in contrast to a large number of nitrogen compounds which either had no effect or stimulated both bacterial growth and phage multiplication.

Schenck & du Vigneaud (153) have demonstrated the synthesis of  $\beta$ -alanine by the growing rat. Fifty times as much  $\beta$ -alanine was found to be deposited in the tissues as was supplied in the diet. While the liver tissue content of  $\beta$ -alanine reflected the amount of pantothenic

acid fed, the extra-hepatic tissues were uninfluenced by dietary intake of  $\beta$ -alanine. The distribution of  $\beta$ -alanine in the different tissues and proteins of the rat was reported by Schenck (154). Skeletal muscle and liver showed the highest content. A number of purified proteins, but none from muscle and liver, were shown to be free of  $\beta$ -alanine.

Pepsin and trypsin hydrolysis of muscle proteins yielded no measurable amounts of carnosine or  $\beta$ -aspartyl histidine (155). Apparently carnosine is not a part of the muscle protein molecule.

Miller (156) has found that *dl*-methionine and *l*-cystine have a protein sparing effect when fed to dogs on a diet containing less than 1 per cent protein. The nitrogen conservation is more marked when the tissue proteins are not severely depleted. The reported protective action of methionine (and cystine plus choline) against hepatotoxins may be explained on the above basis. The protective action of methionine against liver injury by mapharsen when injected into protein depleted dogs has been recently reported by Goodell *et al.* (157). The efficacy of *dl*-methionine in the treatment of acute carbon tetrachloride poisoning in a human is reported by Beattie *et al.* (158).

Glynn & Himsworth (159) observed the production of a massive acute liver necrosis resembling acute yellow atrophy in rats on a protein-deficient diet. Casein and methionine were protective. Yeast protein was found not to be protective due to its low methionine content. [See also (160)].

Ågren (161), in a note, stated that he has further evidence in support of his previous report that the intrinsic factor of Castle is identical with the enzyme aminopolypeptidase.

A polypeptide consisting of 1 mole of *p*-aminobenzoic acid linked to a chain of 10 to 12 glutamic acid residues has been isolated from yeast by Ratner *et al.* (162). A similar compound may also be present in liver, but the low concentration did not permit its isolation.

Christensen (163) has shown that the optically inactive valylvaline isolated from gramicidin hydrolysates (164) was a mixture of *d*-valyl-*d*-valine and *l*-valyl-*l*-valine.

The effect of diet on the histidase, arginase, and urocanase activity of the liver has been studied by Edlbacher & Viollier (165). An increased enzyme activity was noted with rats on a thiamine, riboflavin, or pyridoxin deficient diet. Archibald (120) has applied the carbamido-diacetyl reaction to the determination of citrulline in plasma. The normal fasting level of citrulline was found to be between 0.3 and 1.0 mg. per 100 ml. for man, and between 0.8 and 1.5 mg. per 100 ml. for the dog.

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# THE CHEMISTRY OF THE TRITERPENES

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Because of the large amount of material that has been published since the last review of "The Terpenes, Saponins, and Closely Related Substances" (1), the present review has been limited to the triterpenes. Even for this group of compounds, all of the detailed chemistry on which the arguments for structure are based will not be given but only a summary of the present status of the field.<sup>1</sup> Besides those appearing previously in this publication (1, 2, 3), mention should be made of the excellent reviews of this field by Haworth (4) and by Spring (5).

The triterpenes are natural products containing thirty carbon atoms whose carbon skeleton is believed to be divisible into isopentane units (isoprene rule). They are distributed widely in the plant kingdom and may occur in all parts of the plant, free or combined with sugars. The glycosides constitute one group of the saponins and the aglycones formed on hydrolysis usually are referred to as triterpene sapogenins.<sup>2</sup> Table I lists the sources and molecular formulas of the more extensively investigated triterpenes, while Table II lists some compounds which probably are triterpenes and have been the subject of recent investigations. It is of interest to note that whereas previously all triterpenes, with the exception of the open-chain hydrocarbon squalene, have been obtained from plant sources, lanosterol and agnosterol were isolated first from wool fat where they are associated with the zoosterol, cholesterol (21). Both lanosterol and cryptosterol are present in the complex mixture of phytosterols from yeast (20) and  $\beta$ -amyrin is associated with the phytosterols, stigmasterol and sitosterol, in the dandelion root (50).

Zimmermann (37) has stated that whereas triterpenes have been isolated from all parts of different plants, only in the case of the dandelion have triterpenes been shown to be present in different parts of the same plant. It seems probable that other cases will be found but in

<sup>1</sup> Because of war conditions, foreign literature has been available only for the first few months of 1944.

<sup>2</sup> The other group of saponins on hydrolysis yields steroid sapogenins whose structures are best considered along with those of other steroids.

TABLE I  
MOLECULAR FORMULAS AND SOURCES OF SOME TRITERPENES

Name	Molecular Formula	Source
Agnosterol	$C_{30}H_{48}O$	Wool fat (21)
$\alpha$ -Amyrin	$C_{30}H_{50}O$	Manila elemi resin ( <i>Canarium commune</i> ) and in the resin and latex of many other plants (6); shea nut oil, <i>Butyrospermum (Bassia) parkii</i> (7, 8)
$\beta$ -Amyrin	$C_{30}H_{50}O$	Accompanies $\alpha$ -amyrin
Basseol	$C_{30}H_{50}O$	Shea nut oil (7, 8)
Betulin	$C_{30}H_{50}O_2$	White pigment of birch bark ( <i>Betula alba</i> ) (9)
$\alpha$ -Boswellic acid	$C_{30}H_{48}O_3$	As acetate in olibanum (incense resin) (10)
$\beta$ -Boswellic acid	$C_{30}H_{48}O_3$	Accompanies $\alpha$ -boswellic acid
Cryptosterol	$C_{30}H_{50}O$	Yeast (20)
Echinocystic acid	$C_{30}H_{48}O_4$	As saponin in California manroot ( <i>Echinocystis fabacea</i> ) (11)
Elemadienolic (Elemic, $\alpha$ -Elemolic) acid	$C_{30}H_{48}O_3$	Manila elemi resin ( <i>Canarium commune</i> ) (12)
Elemadienonic ( $\alpha$ -Elemic, $\beta$ -Elemonic) acid	$C_{30}H_{46}O_3$	Accompanies elemadienolic acid
Erythrodiol	$C_{30}H_{50}O_2$	As monostearate in fruit of coca bush ( <i>Erythroxylon novogranatense</i> ) (13)
Glycyrrhetic acid	$C_{30}H_{46}O_4$	As saponin in licorice root ( <i>Glycyrrhiza glabra</i> ) (14)
Gypsogenin	$C_{30}H_{46}O_4$	As saponin in white soaproot ( <i>Gypsophila</i> species) (15) and in fuller's herb ( <i>Saponaria officinalis</i> ) (16)
Hederagenin	$C_{30}H_{48}O_4$	As saponin in ivy leaves ( <i>Hedera helix</i> ) (17), soap nuts ( <i>Sapindus</i> species) (18), leaves of <i>Aralia japonica</i> and of <i>Kalopanax ricinifolium</i> (19)
Lanosterol	$C_{30}H_{50}O$	Wool fat (21) and yeast (22)
Lupeol	$C_{30}H_{50}O$	Frequently associated with the amyryns, e.g., in bresk ( <i>Guttapercha</i> -like material from <i>Alstonia costulata</i> ) (23) and shea nut oil (7)

TABLE I—*Concluded*

Name	Molecular Formula	Source
Oleanolic acid	$C_{30}H_{48}O_3$	As saponin in guaiac bark (24), sugar beet (25), calendula flowers (10), and leaves of <i>Aralia japonica</i> (19) and of <i>Panax japonicum</i> (26) Free in olive leaves (27), clove buds (28), mistletoe leaves (29) and grape skins (30)
Quillaic acid	$C_{30}H_{46}O_5$	As saponin in quillaia bark (31)
Quinovic acid	$C_{30}H_{40}O_5$	As glycoside in <i>Cinchona</i> species (32)
Siarsesinolic acid	$C_{30}H_{48}O_4$	Siam gum benzoin (33)
Sumaresinolic acid	$C_{30}H_{48}O_4$	Sumatra gum benzoin (34)
Ursolic acid	$C_{30}H_{48}O_3$	Widely distributed in the wax-like coatings of leaves and fruits, e.g., apple, cherry, bearberry (35), and cranberry (36)

TABLE II

## PROBABLE TRITERPENES OF RECENT INTEREST

Arnidiol	$C_{30}H_{50}O_2$	Flowers of <i>Arnica montana</i> , colt's foot ( <i>Tussilago farfara</i> ), sunflower ( <i>Helianthus annuus</i> ), and dandelion ( <i>Taraxacum officinale</i> ) (37)
Bassic acid	$C_{30}H_{46}O_5$	As saponin in mowrah meal and shea nut press cake ( <i>Bassia</i> species) (38) and seeds of other Sapotaceae ( <i>Mimusops</i> species, <i>Achras sapota</i> , <i>Dumoria heckelii</i> , <i>Paysonia lucida</i> ) (39)
Brein	$C_{30}H_{50}O_2$	Manila elemi resin (40)
Caoutchicol	$C_{30}H_{50}O$	Jelutong (acetone extract of crude rubber) (41)
Cerin	$C_{30}H_{50}O_2$	Cork (42)
Faradiol	$C_{30}H_{50}O_2$	Accompanies arnidiol (37)
Friedelin	$C_{30}H_{50}O$	Accompanies cerin (42)
Germanicol	$C_{30}H_{50}O$	Dried latex of <i>Lactuca virosa</i> (119)
Gratiolone	$C_{30}H_{48}O_3$	Hedge hyssop ( <i>Gratiola officinalis</i> ) (43)
Maniladiol	$C_{30}H_{50}O_2$	Accompanies brein (40)

TABLE II—*Concluded*

Name	Molecular Formula	Source
Onocerin	$C_{30}H_{48}O_2$	Roots of <i>Ononis spinosa</i> (44)
Pachymic acid	$C_{30}H_{44}O_5$	Bukuryo ( <i>Poria cocos</i> ) (45)
Parkeol	$C_{30}H_{50}O$	Shea nut butter (8)
Platycodigenin	$C_{30}H_{48}O_7$	As saponin in roots of <i>Platycodon grandiflorum</i> (46)
Senegenin	$C_{30}H_{46}O_8$	As saponin in root of <i>Polygala senega</i> (47)
Skimmiol	$C_{30}H_{50}O$	} <i>Skimmia japonica</i> (48)
Skimmione	$C_{30}H_{48}O$	
Soysapogenol-A	$C_{30}H_{50}O_4$	} As saponins in soybeans (49)
Soysapogenol-B	$C_{30}H_{50}O_3$	
Soysapogenol-C	$C_{30}H_{50}O_2$	
Soysapogenol-D	$C_{30}H_{50}O_3$	
Taraxol	$C_{30}H_{46}O_3$	} Roots of dandelion ( <i>Taraxacum officinalis</i> ) (50)
Taraxerol	$C_{30}H_{50}O$	
Taraxasterol	$C_{30}H_{50}O$	
$\Psi$ -Taraxasterol	$C_{30}H_{50}O$	
Vanguerigenin	$C_{30}H_{46}O_3$	As saponin in <i>Vangueria tomentosa</i> (51)
Zeorin	$C_{30}H_{52}O_2$	Lichens (120)

an investigation of various parts of the sunflower plant Zimmermann was able to isolate the triterpene alcohols, faradiol and arnidiol, only from the flower petals, while the same sitosteryl glycoside was isolated from all parts of the plant. In a subsequent paper Zimmermann (52) states that whereas the sterols, steryl glycosides, and paraffin hydrocarbons isolated from fruits and flowers are always the same, the type of triterpene, if present, depends on the type of pigment present. Triterpendiols, he finds, are associated with carotenoid pigments, while triterpene hydroxyacids are associated with anthocyanins.<sup>3</sup>

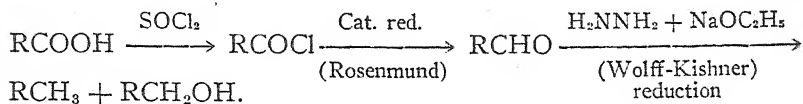
#### INTERCONVERSION OF TRITERPENES

Probably the most important advance in the field of the triterpenes has been the establishment of the suspected relationships between various individual compounds and their assignment to different groups. Because of this work the reactions of each compound in a

<sup>3</sup> Apparently only triterpene hydroxyacids occurring in the free state are being considered since the flowers of *Calendula officinalis*, whose pigments are carotenoid (53), contain a saponin which yields oleanolic acid on hydrolysis (10).

given group have a bearing on the structure of all members of the group and the complete determination of structure for one member will establish the constitution of most members of the group.

These interconversions have depended for the most part on the conversion of a carboxyl group to a methyl group by the following series of reactions:



When an aldehyde or ketone group is present in the molecule, it may be converted to a methyl group or methylene group by reduction. The interconversions that so far have been accomplished may be represented schematically as in Figure 1 (page 388).

From Figure 1 it is clear that not only has a relationship been established between the functional groups of the different compounds but that all have the same carbon skeleton, that the unreactive double bond occupies the same position in all, and that the same stereochemical configurations exist about the numerous asymmetric carbon atoms. An exception to this statement is basseol which has two double bonds, one reactive and one unreactive, and hence has one less ring. The ready cyclization to  $\beta$ -amyrin, however, justifies its inclusion in this group which has come to be known as the " $\beta$ -amyrin group" of the triterpenes.

Not all of the triterpenes can be related to  $\beta$ -amyrin. Thus conversion of the carboxyl groups of ursolic acid (64) and of  $\beta$ -boswellic acid (65) to methyl groups leads in each case to  $\alpha$ -amyrin. A third group is indicated by converting the primary alcohol group of the diol, betulin, into a methyl group and obtaining thereby the monohydric triterpene, lupeol (66). Finally a relationship is suspected between agnosterol, cryptosterol, and lanosterol and the elemi acids (67). It is still too early to decide whether quinovic acid is a representative of still another type of carbon skeleton (117, 118).

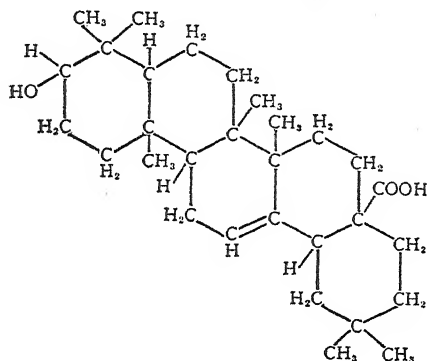
Thus there appear to be at least five different groups of triterpenes: the acyclic group, of which squalene is the only member; the  $\beta$ -amyrin group, the  $\alpha$ -amyrin group, and the lupeol group, all of which are pentacyclic; and the tetracyclic elemi acids group. Aescigenin, the sapogenin obtained by hydrolyzing the saponin from horse chestnuts (*Aesculus hippocastanum*) which formerly was believed to be a triterpene (68), now is thought to be the sole known member of the



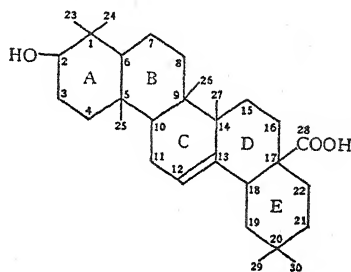
terpenes containing thirty-five carbon atoms in the basic carbon skeleton (69).

### DETERMINATION OF STRUCTURE

*$\beta$ -Amyrin group.*—In order to make the mass of experimental data intelligible, it has been considered best to explain it in terms of one structure for oleanolic acid even though eventually this structure may be displaced by another. The structure which has been defended most staunchly (72, 73) and which admittedly explains a greater number of experimental facts than any other is that first proposed by R. D. Haworth (4) and shown in detail in formula I and in condensed



I. Oleanolic acid



Ia

form in formula Ia with the numbering of the carbon atoms and designation of the rings. The corresponding structures of the other members of the  $\beta$ -amyrin group may be derived from the schematic representation of the interconversions (Fig. 1) in which the carbon atom of the upper group in the brackets is C-23 and that in the lower group is C-28.

As was pointed out in the previous review (1) the assignment of a hydrogenated picene nucleus is based primarily on the structure of the aromatic hydrocarbons formed on dehydrogenation with selenium at relatively high temperatures (320 to 350°). The different distribution of side chains and double bond in the present formulation as compared to that given in the previous review does not affect the interpretation of the products of selenium dehydrogenation.

The structure of ring A and the portion of ring B through C-7 has been proven rigidly by stepwise oxidative degradation of heder-

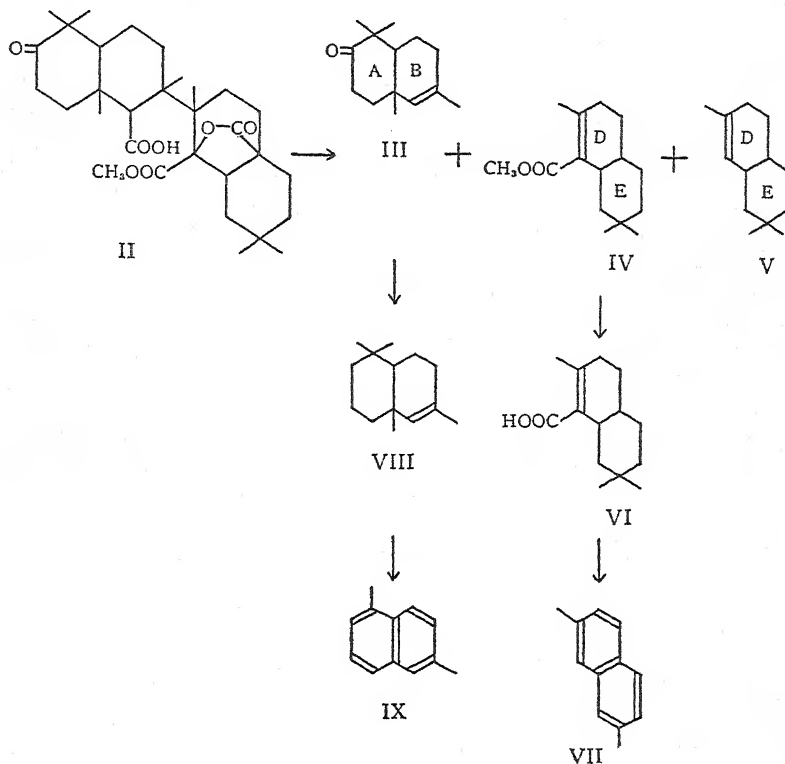


agenin and oleanolic acid (1, 2). The evidence presented by Kitasato (74) that the carboxyl group is located at C-5 rather than at C-17 has been shown to be in error (75). The oxidation product which supposedly contained a malonic acid grouping has two carboxyl groups in the 1,6-positions with respect to each other.

The location of the double bond in ring C is based on the pyrolysis of the monomethyl ester of isooleanonic lactone dicarboxylic acid (II) which is formed from oleanolic acid by a series of reactions including oxidative fission of the ring containing the double bond (72). The product of pyrolysis contained a ketonic fraction which was separated by Girard's reagent T and gave a semicarbazone of the ketone  $C_{14}H_{22}O$  (III). The presence of an ester portion (IV) was shown by a methoxyl determination and saponification to an acid  $C_{14}H_{22}O_2$  (VI). A hydrocarbon fraction  $C_{13}H_{22}$  (V) also was obtained. The ketone was reduced to the hydrocarbon VIII which gave 1,6-dimethylnaphthalene (IX) on dehydrogenation with selenium while both the acid VI and the hydrocarbon V gave 2,7-dimethylnaphthalene (VII). The isolation in approximately equal amounts of the two fractions "tagged" respectively with the ketone group and the carbomethoxy group indicates that the molecule has been split into halves, and the characterization of these fractions as hydrogenated naphthalene derivatives confirms the supposition that ring C was opened on oxidation. The conversion of the ketone into 1,6-dimethylnaphthalene and the acid into 2,7-dimethylnaphthalene is in conformity with the assumed distribution of the methyl groups.

The conversion of oleanic acid into a lactone and bromolactone in which the hydroxyl group is not involved (10, 29) indicates that the carboxyl group is  $\beta$ ,  $\gamma$ , or  $\gamma,\delta$  to the double bond. The decision to locate the carboxyl group at C-17 and the double bond between C-12 and C-13 rests on a number of reactions involving rings D and E. Oxidation of methyl acetyloleanolate with selenium dioxide in boiling acetic acid solution gave two compounds,  $C_{33}H_{50}O_4$  and  $C_{33}H_{46}O_6$  (70). The latter compound on the basis of the absorption spectrum ( $\lambda_{max.} = 275$  m $\mu$ ,  $\log \epsilon = 4.1$ ) is formulated as a diendione (X) (76). Whereas methyl oleanolate is resistant to hydrolysis, the ester group of the diendione can be saponified with alcoholic potassium hydroxide, presumably because it is a vinylogue of a  $\beta$ -ketoacid, and the acid so obtained (XI) on heating in xylene loses carbon dioxide. The resultant neutral product is assigned the rearranged structure XII because of the marked change in the absorption spectrum ( $\lambda_{max.} = 245$ ,  $\log \epsilon = 4.25$ ). Re-

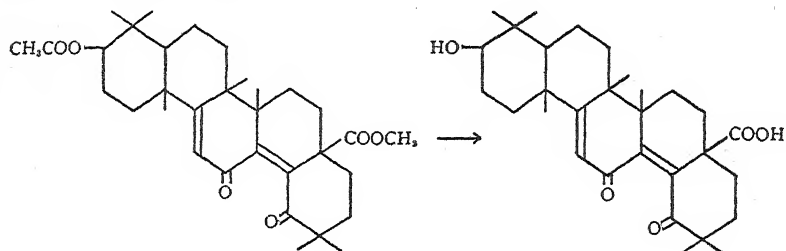
action of XII with hydrazine leads to the formation of a pyridazine (XIII) which requires a 1,4-position for the carbonyl groups and a spatial arrangement that will lead to a strainless six-membered ring.



Further oxidation of the original diendione (X), with chromic anhydride gives a neutral product,  $C_{33}H_{46}O_7$ , containing one more oxygen atom than the original compound. Alkaline saponification at  $200^\circ$  causes the loss of a carbon atom and gives an acid,  $C_{29}H_{44}O_5$ . The new noracid gives a positive color reaction with ferric chloride indicating a 1,3-diketone grouping. These reactions are interpreted by formulas XIV and XV.

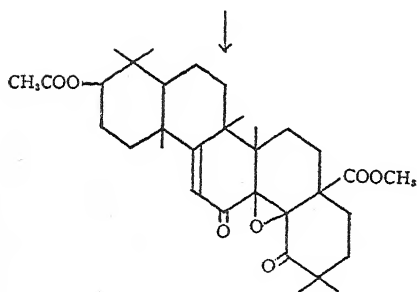
Assuming that formula I represents the constitution of oleanolic acid, the structures of  $\beta$ -amyrin,  $\alpha$ -boswellic acid, erythrodiol, gypso-genin, and hederagenin follow directly from the interconversions. These conclusions are confirmed by reactions of the compounds analogous to those for oleanolic acid. For example  $\beta$ -amyrin acetate on

energetic oxidation with selenium dioxide (77) gave a diendione which has a methyl group in place of the carbomethoxy group. The diendione gave with hydrazine a pyridazine, while oxidation gave an oxide which on saponification gave an acid. The structure of the diendione and derived products may be represented by replacing the carbomethoxy group or hydrogen at C-17 by a methyl group in formulas X, XIII, XIV, and XV (78). The absorption spectra of the respective analogues were the same.

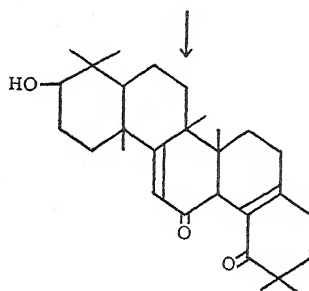


X

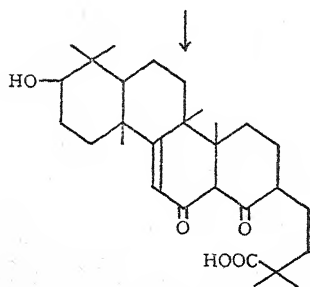
XI



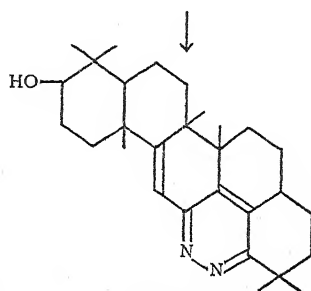
XIV



XII



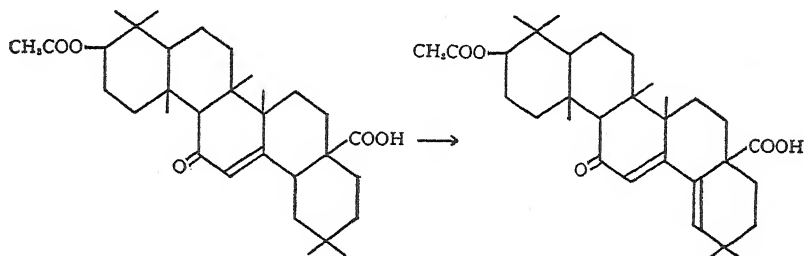
XV



XIII

For the remainder of the members of the  $\beta$ -amyrin group, the assignment of structure is not so direct because of the presence of additional functional groups. Glycyrrhetic acid has, in addition to hydroxyl and carboxyl, a carbonyl group which is conjugated with the double bond (79) and hence is definitely located at C-11. On catalytic hydrogenation, two moles of hydrogen are absorbed and the carbonyl group is removed leaving the double bond intact (80). The deoxoglycyrrhetic acid is isomeric but not identical with oleanolic acid. Since conversion of the carboxyl groups of both acids to methyl groups gives  $\beta$ -amyrin (61), deoxoglycyrrhetic acid must differ from oleanolic acid either in the configuration of the carbon atom to which the carboxyl group is attached, or in the location of the carboxyl group.

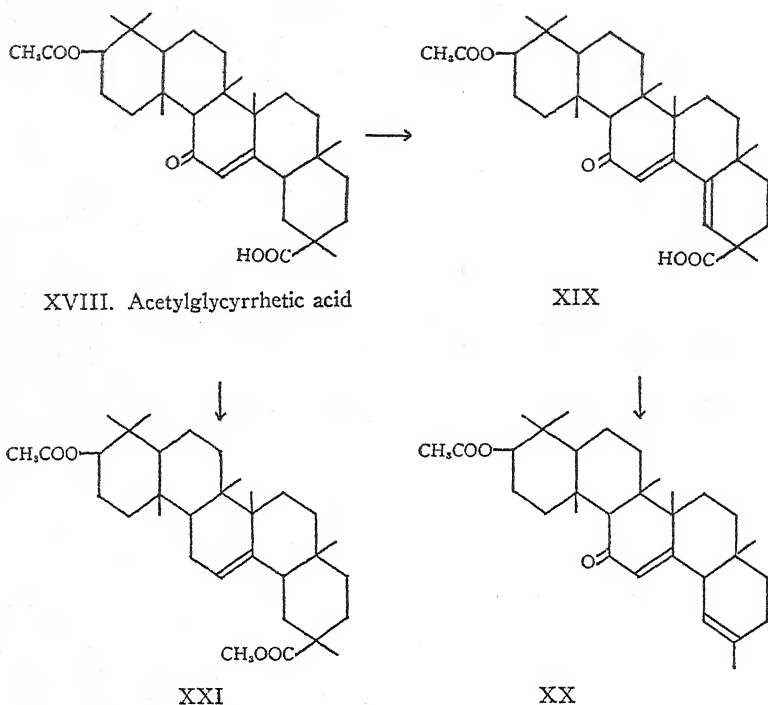
Acetylketooleanolic acid (XVI), formed as one of the products of chromic anhydride oxidation of acetyloleanolic acid (81), on treatment with bromine loses two hydrogen atoms with the formation of a new double bond which was shown to be in conjugation with the original  $\alpha,\beta$ -unsaturated carbonyl system ( $\lambda_{\max.} = 284 \text{ m}\mu$ ,  $\log \epsilon = 4.05$ ) (76). The acid can be sublimed at reduced pressure without decomposition at 260 to 270° and is assigned formula XVII. Acetyl-



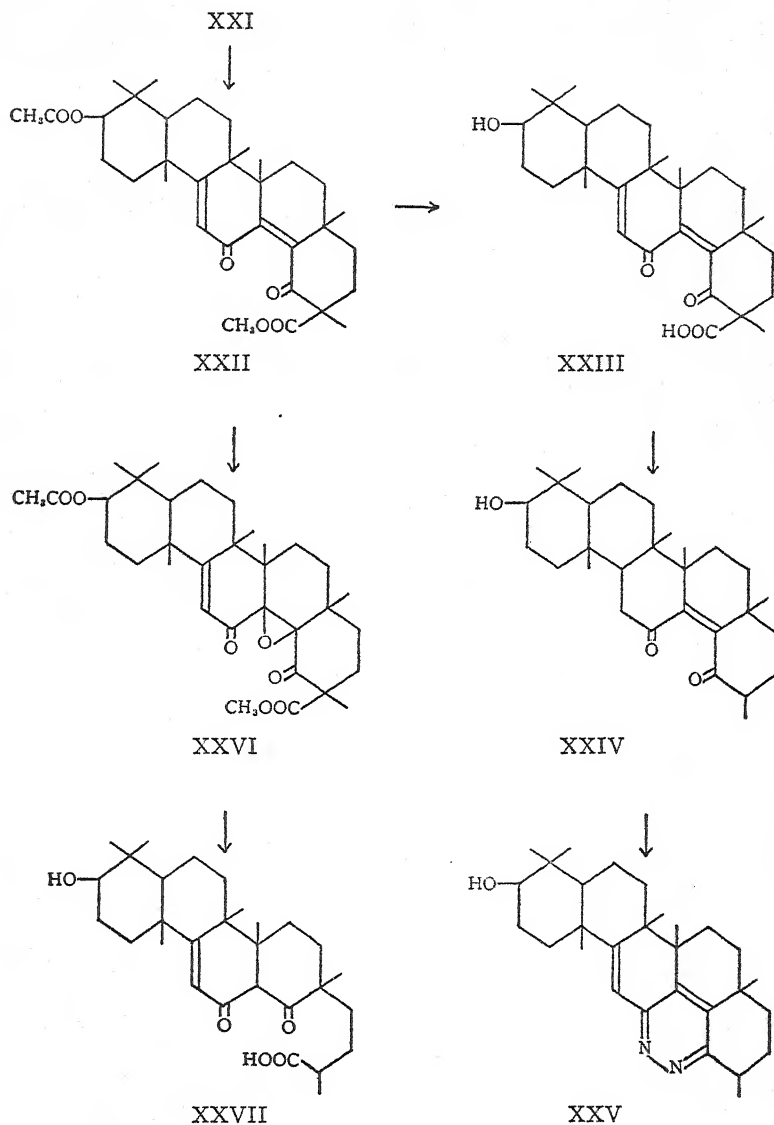
XVI. Acetylketooleanolic acid

XVII

glycyrrhetic acid, which is isomeric with acetylketooleanolic acid, gives an analogous compound on dehydrogenation with bromine but the product loses carbon dioxide at 205 to 210° (82). The absorption spectrum of this decarboxylated compound ( $\lambda_{\max.} = 250 \text{ m}\mu$ ,  $\log \epsilon = 4.35$ ) indicates the presence of a simple  $\alpha,\beta$ -unsaturated ketone group in contrast to the completely conjugated doubly unsaturated ketone grouping present in the dehydro acid ( $\lambda_{\max.} = 280 \text{ m}\mu$ ,  $\log \epsilon = 4.0$ ), so decarboxylation must have been accompanied by a shift of one double bond. This difference in behavior can be explained by placing the carboxyl group of glycyrrhetic acid at C-20 (XVIII). The dehy-



droacid then is represented by XIX and the decarboxylated product by XX. Further evidence that the carboxyl group cannot occupy the same position in oleanolic acid as in glycyrrhetic acid has been given. Methyl acetyldeoxoglycyrrhetate has been put through the same series of reactions (XXI to XXVII) as methyl acetyloleanolate (X to XV). Whereas the acid XI lost carbon dioxide only on heating in xylene, acid XXIII loses carbon dioxide during saponification in alcohol solution (82) as might be expected of a  $\beta$ -ketoacid. Moreover, in agreement with the assumption of a methyl group at C-17 (XXIV), no shift of the double bond occurs, as happens on decarboxylation of the oleanolic acid derivative (XII). The diketoxide (XXVI), like XIV, loses carbon dioxide on drastic alkaline saponification to give a nordiketoid (XXVII) isomeric with XV. Hence oleanolic acid and deoxoglycyrrhetic acid cannot both have their carboxyl groups at C-20 and differ in the configuration of this carbon atom, since the conversion of XXVI to XXVII must proceed

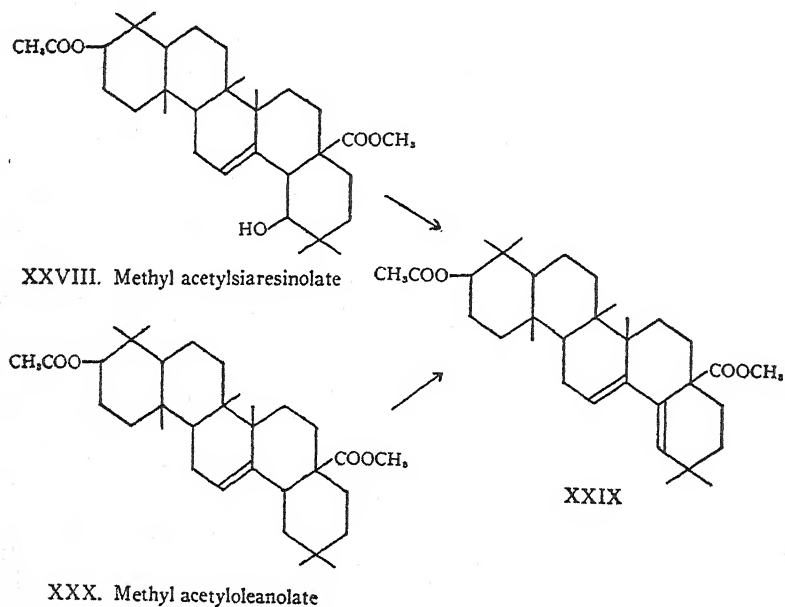


through a malonic acid derivative and the asymmetry of C-20 would be destroyed, in which case XXVII would be identical with XV.

As indicated previously, quillaic acid differs from echinocystic acid only in that it has an aldehyde group replacing a methyl group (62,83).

Since the secondary hydroxyl common to all triterpenes of the  $\beta$ -amyrin group and the aldehyde group occupy positions 1,3 with respect to each other (31) the aldehyde group can be located only at C-1. Echinocystic acid is a  $\beta$ -hydroxyacid (84) so the second hydroxyl group must occupy position 16 or 22. Measurement of the area of surface films of the compound having no oxygen-containing groups other than this hydroxyl indicated that it was located at C-16 (85) if the carboxyl group in the original compound is at C-17.

Siaresinolic acid and sumaresinolic acid likewise have an additional secondary hydroxyl group. In both, one hydroxyl is less readily esterified than the other. When the monoacetyl methyl ester of siaresinolic acid (XXVIII) is dehydrated with phosphorus pentoxide in benzene (71), the product (XXIX) is identical with that formed by oxidation of methyl acetyloleanolate (XXX) with selenium dioxide (70) and hence the second hydroxyl is assigned to C-19 (86). While no at-



tempted interconversion of sumaresinolic acid has been successful, it undergoes numerous reactions to give products analogous to those obtained from members of the  $\beta$ -amyrin group and is believed to belong to this group. Oxidation of the monoacetyl methyl ester with

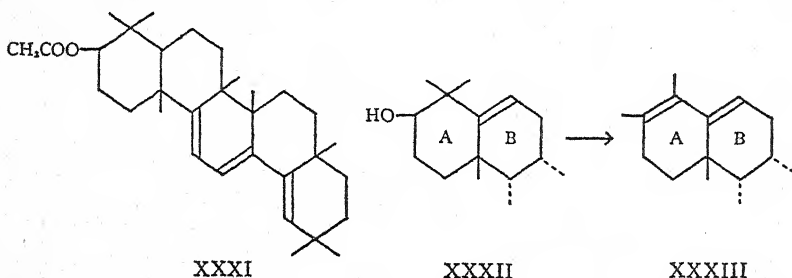
chromic anhydride gives the monoacetyl monoketo methyl ester which after conversion to the lactone can be oxidized with selenium dioxide to an enolizable 1,2-diketone ( $\lambda_{\text{max.}} = 293$ ,  $\log \epsilon = 4.03$ ). The grouping  $-\text{CHCOCO}-$  could not be accommodated anywhere except in ring B and hence the hydroxyl group in question is assigned to position 7 or 8 (87).

Before leaving this group of triterpenes it should be emphasized that reference to much valuable work has been omitted and controversial points ignored in order to present a comprehensible picture in the space allotted. The formulations given, however, do not account for a number of observations. For example, measurements of the areas of surface films of oleananic and hedraganic acids, in which all functional groups except the carboxyl group have been eliminated, give values which are much too small for a hydrogenated picene nucleus having a carboxyl group at C-17 but which agree with the expected value if the carboxyl group is in one of the end rings of such a nucleus (83). Recently the position of the absorption maximum for conjugated dienes (88, 89) and for  $\alpha,\beta$ -unsaturated ketones (90, 91) has been correlated with the environment of the unsaturated system. Although numerous conjugated dienes and  $\alpha,\beta$ -unsaturated keto derivatives of the triterpenes have been prepared and their absorption spectra determined, in no case does the position of the absorption maximum agree with the value predicted from the formula assigned to any of the compounds (92). Moreover, the cyclization of basseol to  $\beta$ -amyrin (55) and the easy saponification of methyl glycyrrhetate and difficult saponification of the deoxoester (80) are inexplicable on the basis of the formulas assigned to them.

*$\alpha$ -Amyrin group.*— $\alpha$ -Amyrin is isomeric with  $\beta$ -amyrin and remarkably like it in its chemical reactions. Both contain one secondary hydroxyl group (93) and yield the same products on dehydrogenation with selenium (94, 95). Both contain a double bond which cannot be hydrogenated but the double bond of  $\beta$ -amyrin forms an oxide with perbenzoic acid whereas that of  $\alpha$ -amyrin does not (96). As in the case of  $\beta$ -amyrin, chromic anhydride oxidation introduces a carbonyl group conjugated with the double bond (95) from which  $\alpha$ -amyrin can be regenerated by catalytic reduction (80). Reduction of both  $\alpha,\beta$ -unsaturated ketones with sodium and alcohol followed by dehydration gives dienols, the absorption spectra of which show that the double bonds are conjugated in a single ring (97). From these reactions



one may conclude that the ring containing the double bond in  $\alpha$ -amyrin must contain the grouping  $-\overset{|}{\text{CH}}-\text{CH}_2-\overset{|}{\text{CH}}=\overset{|}{\text{C}}-$ . This structure is present in ring C of formula I but other reactions make it seem impossible for  $\alpha$ -amyrin to be merely a stereoisomer of  $\beta$ -amyrin. Besides the fact that  $\alpha$ -amyrin does not form an oxide with perbenzoic acid, it cannot be oxidized to a dehydroderivative analogous to compound XXIX as can  $\beta$ -amyrin (98). Moreover, while  $\beta$ -amyrin acetate is oxidized by N-bromosuccinimide to the completely conjugated triene, XXXI ( $\lambda_{\text{max.}} = 308 \text{ m}\mu$ ,  $\log \epsilon = 4.1$ ), the same reagent introduces only one double bond into  $\alpha$ -amyrin acetate (99) to give the previously known diene in which both double bonds are conjugated in a single ring (97). Methyl acetylursolate behaves similarly to  $\alpha$ -amyrin on oxidation with N-bromosuccinimide. Further evidence against the location of the original double bond in ring D is the dehydration of  $\alpha$ -amyrin with phosphorus pentoxide to a diene having the two double bonds conjugated in two rings ( $\lambda_{\text{max.}} = 239.5$ ,  $\log \epsilon = 4.2$ ) (100). Hence the original double bond must have been near the hydroxyl group, which is believed to occupy the 2-position since the same 1,5,6-trimethyl- $\beta$ -naphthol is obtained on selenium dehydrogenation of both  $\alpha$ - and  $\beta$ -amyrins (94, 95). Accordingly it has been suggested that the double bond is in the 6,7-position and that dehydration takes place with a retropinacolone rearrangement (partial formulas XXXII and XXXIII). However, if the double bond is in the 6,7-position it is

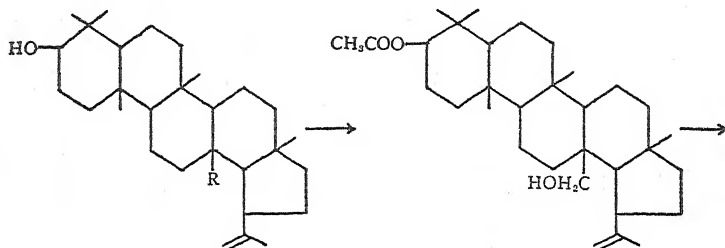


not possible to have a methyl group at C-9 since the formation of the dienol with the double bonds conjugated in a single ring (97, 99) requires the system  $\text{C}=\text{CH}-\text{CH}_2-\text{CH}-$ . Because the mechanism postulated to explain the formation of 1,5,6-trimethyl- $\beta$ -naphthol and 1,2,5,6-tetramethylnaphthalene on selenium dehydrogenation requires a methyl group at C-9, it is not possible at present to give a rational formulation for the  $\alpha$ -amyrin group.

*Lupeol group.*—Betulin,  $C_{30}H_{50}O_2$ , contains one double bond, one secondary hydroxyl group, and one primary hydroxyl group and hence is pentacyclic (102). It has been converted into lupeol,  $C_{30}H_{50}O$ , by converting the primary alcohol group into a methyl group (66). In contrast to the double bonds in the  $\alpha$ - and  $\beta$ -amyrin groups, that in lupeol can be readily hydrogenated (101). Ozonolysis of the acetate gives formaldehyde so that the double bond is exocyclic (103). Oxidation of lupeol acetate with selenium dioxide gave an  $\alpha,\beta$ -unsaturated aldehyde rather than an  $\alpha,\beta$ -unsaturated ketone and since lupeol can be regenerated by Wolff-Kishner reduction, it is unlikely that rearrangement of the double bond has taken place before oxidation and the methylene group must be present in an isopropenyl side chain rather than attached to the ring (104). As a working hypothesis formula XXXIV has been proposed for lupeol and betulin (104, 105) on the basis of the products of selenium dehydrogenation. Rings A and B are formulated as in the case of the  $\beta$ -amyrin group because dehydrogenation products assumed to be derived from these rings are obtained from both groups. No products indicating a hydrogenated picene structure have been obtained from lupeol and to account for this fact ring E has been assumed to be five-membered. The production of 2,7-dimethylnaphthalene and 1,2,7-trimethylnaphthalene from betulin, products believed to arise from rings D and E in the  $\beta$ -amyrin series, is assumed to be due to ring enlargement and migration of a carbon atom during the dehydrogenation (104). The relative position of the primary alcohol group and the isopropenyl group in betulin is based on the following series of reactions: chromic anhydride oxidation of betulin monoacetate (XXXV) at 20° gave acetylbetulinic acid (XXXVI) (106), which on oxidation with selenium dioxide gave the unsaturated aldehyde (XXXVII). Further oxidation with chromic anhydride gave the bisnordicarboxylic acid (XXXVIII) which could be converted to the anhydride (XXXIX) (105).

*Elemi acids group.*—Two acids, which in the past usually have been known as  $\alpha$ -elemolic acid and  $\beta$ -elemonic acid, are characteristic of Manila elemi resin. The separation of the acids and their isolation in a pure state has been facilitated by the use of Girard's reagent T (107) or reagent P (108) to remove the ketoacid. Both acids contain two double bonds and only one double bond in each acid is reducible catalytically. Both acids on ozonolysis or oxidation with chromic anhydride yield acetone and a new acid containing three less carbon

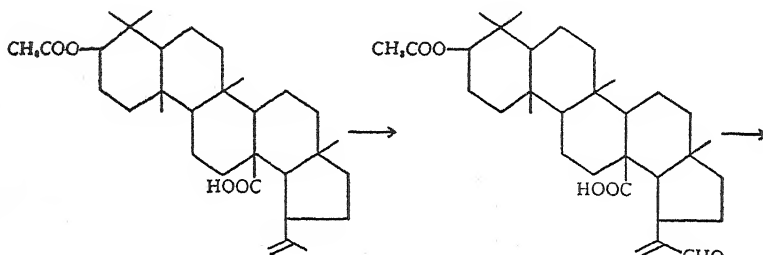
atoms (109) so that the reactive double bond must be present in the grouping  $-\text{CH}=\text{C}(\text{CH}_3)_2$ . From the molecular formulas and the number of double bonds, the acids must be tetracyclic in contrast to the amyrin groups which are pentacyclic. However, unlike basseol, the elemi acids cannot be cyclized by formic acid (108, 110). Since



XXXIV

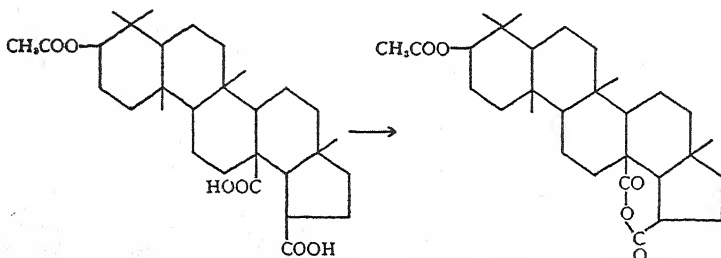
Lupeol,  $\text{R}=\text{CH}_3$ Betulin,  $\text{R}=\text{CH}_2\text{OH}$ 

XXXV



XXXVI

XXXVII



XXXVIII

XXXIX

$\alpha$ -elemolic acid on oxidation with chromic anhydride gives a mixture of  $\alpha$ -elemonic acid and  $\beta$ -elemonic acid (109) and  $\beta$ -elemonic acid on reduction with hydrogen and Raney nickel catalyst gives a mixture of dihydro- $\beta$ -elemolic acid and dihydro- $\alpha$ -elemolic acid, a close relationship must exist between  $\alpha$ -elemolic and  $\beta$ -elemonic acids. It has been concluded (111) that  $\alpha$ -elemolic acid is indeed the alcohol corresponding to  $\beta$ -elemonic acid and that  $\alpha$ -elemonic acid and  $\beta$ -elemonic acids are isomeric compounds.  $\beta$ -Elemolic acid is considered to be the epimer of  $\alpha$ -elemolic acid in which the secondary hydroxyl group occupies the epimeric position, while  $\alpha$ -elemonic acid is formed by the rearrangement of the two isolated double bonds to a conjugated position. Accordingly a new system of nomenclature has been devised in which the various derivatives are divided into two groups, the normal series, in which the double bonds are separated, presumably by a single carbon atom, and the iseries, in which they are conjugated. In this system of nomenclature  $\alpha$ -elemolic and  $\beta$ -elemonic acids, which both belong to the normal series, become elemadienolic and elemadienonic acids, respectively, while  $\alpha$ -elemonic acid becomes isoelemadienonic acid and  $\beta$ -elemolic acid becomes epielemadienonic acid.

Lanosterol,  $C_{30}H_{50}O$ , and agnosterol,  $C_{30}H_{48}O$ , contain two and three double bonds respectively and hence are tetracyclic. Only one double bond in each can be hydrogenated and two of the double bonds in agnosterol are conjugated (21, 112). Dihydrolanosteryl acetate can be oxidized to an unsaturated ketone which after reduction to the unsaturated alcohol and dehydration yields agnosteryl acetate (113, 114). Lanosterol and cryptosterol,  $C_{30}H_{50}O$ , apparently yield the same acid,  $C_{25}H_{40}O_3$ , on oxidation with hydrogen peroxide and osmium tetroxide (115). It is more likely that this acid has the molecular formula  $C_{27}H_{44}O_3$ , since it seems to be identical with an acid of this composition obtained by the ozonolysis of lanosterol (67). These compounds not only are closely related to each other but their chemical behavior indicates that they are related to the elemi acids. Not only are they tetracyclic with one double bond that can be hydrogenated by catalytic reduction, but this double bond is present in an isopropylidene group since ozonolysis of lanosterol or cryptosterol gives acetone (22, 67). Moreover, the principal product of dehydrogenation with selenium is 1,2,8-trimethylphenanthrene (116, 67) as is the case with  $\alpha$ -elemolic acid (109), and analogous compounds are obtained by chromic anhydride oxidation of dihydro- $\alpha$ -elemolic acid and dihydrocryptosterol (109, 115). Attempts, however, to relate the

two groups of compounds more closely by comparing hydrocarbons derived from them have not been successful (67). In view of the demonstrated nonrelationship to the sterols, it would seem to be advisable to change the names of lanosterol, agnosterol, and cryptosterol, possibly to lanol, agnol, and cryptol.

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## MINERAL METABOLISM

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The term "mineral metabolism" embraces aspects of biochemistry and physiology so broad in scope and so detailed as to make this review of the subject necessarily both arbitrary and limited. The writer has accordingly availed himself of the liberal editorial policy governing these reviews to include a consideration of recent developments bearing on the chemical and physiological activities not only of metallic elements or salts, but of certain acid-base metabolites. In some respects, then, this review may be regarded as supplementary not only to the preceding review (1) but also to those of McLean (2), Shohl (3), and Sendroy (4). Thus, this treatment necessitates the arbitrary selection of topics of established or increasing importance and interest, with the limitations imposed by the space available and by the desirability of avoiding an overlapping of the many closely related fields of study covered by other reviewers. Attention is directed to the contributions appearing in the literature for nearly all of 1944 and also to some work published since the author's previous summary (4) and not covered in other recent reviews (5, 6, 7). Insofar as possible, strictly nutritional aspects of mineral metabolism are omitted, in the expectation that they will be covered in an accompanying article (8) of this volume.

### NOMENCLATURE

Inasmuch as our understanding of the processes involved in mineral metabolism has developed to a large extent, *pari passu*, with our ability to apply fundamental physicochemical principles to the particular problem at hand, a few remarks on recent theoretical developments and the treatment of data may be appropriate. In the maintenance of the steady state called "homeostasis" (9) the metabolic activities of the "mineral" substances take place through chemical reactions in all three physical phases. While some of the systems involved may be far from true equilibrium, the laws of chemical equilibrium, as has been shown by McLean (10), may advantageously be applied, especially to the study of the liquid, or intermediary phase.

Although the transition from Arrhenius's fundamental concept of

electrolytic dissociation to the modern treatment of Bjerrum, Brönsted, Debye & Hückel, Noyes, and others has served to shed light on many biological problems, there still exists in some respects what may seem to be an apparent state of confusion among workers. Thus, for example, in accordance with the assumption of complete dissociation of salts, in recording formulae and equations it would seem desirable to recognize the reactivity of such substances in solution as being that of the individual ions,  $\text{Na}^+ + \text{HCO}_3^-$ ,  $\text{K}^+ + \text{Cl}^-$ , rather than that of the molecules  $\text{NaHCO}_3$  and  $\text{KCl}$ , which represent analytical relations. Even more pertinent to any discussion of mineral metabolism from the standpoint of physiological, chemical, or electrical neutrality, is the varied and interchangeable use, in biochemistry, of the words "base" and "alkali" to mean (a) a cation,  $\text{B}^+$ , which balances an anion,  $\text{A}^-$ , electrochemically in solution; (b) B, the metallic part or radicle of a salt which may or may not completely yield  $\text{B}^+$  ions; (c)  $\text{BOH}$ , a substance which neutralizes acids by furnishing  $\text{OH}^-$  ions to neutralize  $\text{H}^+$  ions; and (d) bicarbonate in biological fluids, representing an equivalent amount of  $\text{B}^+$  or B "available" for the neutralization of acids other than carbonic. While all of these usages are conventionally well understood by most workers in any particular application, they cannot all satisfy the requirements of correct chemical terminology. Some progress has already been made in correcting this condition (3, 10), which awaits more complete clarification in the future. These remarks are prompted by difficulties arising in the writer's own experience, confirming that of Dr. Wm. Mansfield Clark, who has for some time past called attention to the need for a revision of our nomenclature (11).

#### ACID-BASE BALANCE AND METABOLISM

*Physicochemical constants in carbonic acid chemistry.*—As aids in the elucidation of acid-base metabolism data the following physicochemical studies have been recorded. The earlier values for the first and second dissociation constants of carbonic acid in water at  $38^\circ$  (12) have again been substantially confirmed in Harned's laboratory (13, 14), as has the constant (15) for the solubility coefficient of carbon dioxide at  $38^\circ$  (13, 16). Rossier & Méan (17), using both hydrogen and glass electrodes, have confirmed the now well-established value of 6.10 (18, 19, 20) for the  $\text{pK}'_1$  of human plasma at  $38^\circ$ . Agreement with earlier values for  $\text{pK}'_1$  of carbonic acid in red blood cells (20, 21, 22) has been found by Danielson, Chu & Hastings (23) for oxygenated ox

cells at 38° ( $pK'_1 = 6.09$ ).<sup>1</sup> From these results, coupled with those obtained for concentrated egg albumin and muscle protein solutions, they concluded that tissue protein, *per se*, has no effect on the value of  $pK'_1$  for carbonic acid. Their results indicate that when concentrations are calculated as moles per kilo of water, one may reasonably assume, unless proved otherwise, that  $pK'_1$  of all tissues at 38° will not be sensibly different from the value 6.10.

*Kinetics of carbonic acid chemistry.*—Of importance for the physiological transport of carbon dioxide in the body, are studies of the kinetics of carbonic acid, with especial reference to its hydration (24, 25). It has been shown that various inorganic catalysts, in the form of buffer salts such as sulfite and selenite (26, 27), and, to a greater extent, bromine and hypobromous acid (28), have a strong catalytic effect on the hydration of carbon dioxide. Thus, the possible effect of the inorganic constituents of the solution must be taken into consideration in all studies of carbonic acid kinetics, particularly those involving the activity of carbonic anhydrase.

*Standardization of the pH scale.*—This subject, involving uncertainties in theory and method, still claims attention (29, 30) as improvements are made to provide standards of reference adjusted to overcome theoretical limitations to the extent required by present day measurements (31, 32). Provisionally, at least, the values given by MacInnes, Hitchcock, Hamer, and their associates for reproducible, clearly defined reference systems are available as the standard of reference for pH measurements (31 to 34). In such systems, buffer mixtures have replaced the use of 0.1 *N* hydrochloric acid, an unfortunate medium of standardization adopted at a time, about two decades ago, when newer developments in the theory of solutions promised a closer approach of practice to theory in what were then assumed to be measurements of hydrogen-ion activity (4, pp. 232–33).

*Glass electrode.*—The usefulness of this type of electrode is increasing, and with it, an awareness of its limitations (34). However, despite its shortcomings, which for most practical purposes are far outweighed by its advantages, the glass electrode represents one of the most outstanding recent additions to the armamentarium of the biological investigator. The monograph by Dole (35) on the glass electrode is timely, important, and well done.

<sup>1</sup> Values of  $pK'_1$  referred to here are calculated without regard to the state of hydration of carbon dioxide or the amount of its carbamino compound present [(4), p. 236].

*Temperature coefficient of blood pH.*—Skotnický (36) has reported measurements of the change in pH with temperature for venous blood and serum over the range of 17° to 37°. The temperature coefficients of  $-0.017$  pH per degree for normal or pathological human blood, and for sheep blood, and higher values of  $-0.021$  and  $-0.023$  pH per degree for human and beef serum, respectively,<sup>2</sup> are of the same order of magnitude as the total  $c$  correction found for Cullen's (37) colorimetric serum pH method (38). Although Robinson & Hogden (39) have shown that the adsorption of the phenol red indicator by protein will influence the magnitude of the correction, inasmuch as the temperature coefficient is the major component of this correction, there would seem to be reasonable justification in the method of Hastings & Sendroy (40) for the elimination of this coefficient in correlating colorimetric and electrometric serum pH values.

*Variation in resting normal arterial blood pH.*—The pH of the serum of arterial blood in normal young males under basal conditions has been determined by the gasometric method, for which a sensitivity or "range of error" of  $\pm 0.003$  pH is claimed by d'Elseaux *et al.* (41). The authors conclude that basal fluctuations in the arterial serum pH of an individual, and in the variations of the means of pH values among individuals, are less than 0.01 of a pH unit. If their treatment of the data is correct, these results indicate a narrower range of variation of arterial blood pH than has hitherto been reported for any physiological condition. To the extent that the above results are based on the analyses of three different blood samples drawn simultaneously from the same artery, they are inadequate as a study of fluctuation within the same individual. However, four subjects tested several months later did show, within the limits cited, an unchanged arterial blood pH. In any case, in the absence of the various stimuli associated with normal body activity, the physiological maintenance of a constancy of serum hydrogen ion concentration within the limits of  $\pm 1.25$  per cent (at pH 7.4), in a given individual at the same level of metabolic activity, may be accepted as reasonable. On the other hand, the observation of a like constancy of serum pH within the entire range of individuals is of such physiological importance that it should be verified in other laboratories.

<sup>2</sup> The difference between  $dpH/dT$  for human blood and serum indicates that the values given apply to separated serum. For true serum, since the pH of blood is that of the plasma (or serum) bathing it, the temperature coefficient would be the same as for blood.

That such constancy is not maintained throughout the day, even in the resting, normal individual, was found by Hastings & Eisele (42), who studied the acid-base balance changes in the blood of a male human subject during two twenty-four hour periods, one week apart. The arterial serum pH of samples taken at two hour intervals, fluctuated between 7.31 and 7.43 in the first experiment and between 7.36 and 7.42 in the second. An observed trend toward an increase in the basic blood component, indicated by a rise in serum  $\text{HCO}_3^-$ , usually took place after meals and was ascribed to the combined effect of the digestive process and the food content. Carbon dioxide tension, on the other hand, showed little variation during the day but increased over night during sleep. In general, this was paralleled by the serum pH, which was maintained between 7.40 and 7.43 during the day and decreased in the evening and over night to the lowest values.

*Internal erythrocyte pH.*—Direct measurement of the pH within the intact red blood cell (43) by a spectrophotometric method in which methemoglobin serves as the indicator, now appears feasible. The values to be found under physiological conditions are awaited with interest.

*Intracellular acid-base balance of muscle.*—The mean intracellular pH of cat skeletal muscle has been calculated by Wallace & Hastings (44) to be  $6.93 \pm 0.12$ . Their conclusion that the intracellular bicarbonate concentration is constant and relatively unaffected by large changes in the bicarbonate concentration of the extracellular fluid, is in agreement with previous work indicating the general impermeability of muscle cells to anions. It appears that intracellular muscle pH is governed largely by changes in carbon dioxide tension, a conclusion which throws new light on the role of the tissues and their relationship to body fluids in the regulation of physiological neutrality. Thus, the authors point out that a condition of alkalosis accompanied by an increased carbon dioxide tension may be characterized by an intracellular acidosis. These *in vivo* studies have been confirmed by *in vitro* experiments (45).

*Acid-base balance of pancreatic and intestinal secretions.*—Strong evidence that the bicarbonate of pancreatic juice is derived mainly from the plasma and not from the metabolic carbon dioxide of the gland itself, has recently been furnished by studies in which three different experimental approaches were employed. Montgomery, Sheline & Chaikoff (46) found that radioactive sodium injected intravenously into dogs appeared in the pancreatic secretion within three minutes, the

concentrations of labeled sodium in the juice closely following those of the serum. It had already been shown (47) that changes of serum sodium and potassium cation concentration were promptly reflected in a corresponding change in the juice. Similarly, Ball *et al.* (48) gave injections of bicarbonate containing radioactive carbon to dogs and found a prompt appearance of the labeled bicarbonate anion in the juice in a concentration four to five times that in the serum, corresponding to the ratio of the total carbon dioxide concentration in juice to that in serum. It is estimated that under the conditions of these experiments, no more than 20 per cent of the pancreatic secretion of bicarbonate could have originated from metabolic carbon dioxide. The authors found the chloride content of actively secreting pancreatic tissue to be the same as that of the resting gland. Although the total carbon dioxide of the former was somewhat higher than that of the latter, this disproportion is reasonably ascribed to contamination of the active gland by juice of high total carbon dioxide content. Oldfelt (49) has apparently overcome this difficulty by measuring the carbon dioxide binding capacity of suspensions of pancreatic tissues taken from cats with glands in the resting and profusely secreting states. The alkali reserve, and likewise the sum of the cations  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$ , were found to be the same for the excised glands before and after secretion, indicating that the bicarbonate of the secretion must have been derived from the blood.

In work which is consistent with observations by others on dogs, human jejunal juice has been found by McGee & Hastings (50) to have an acidity within the limits of pH of  $6.5 \pm 0.3$  and an average carbon dioxide tension of  $100 \pm 50$  mm. It is concluded that the high carbon dioxide tension of the secretion is the result of the specific secretory processes involved in its formation. The response to a displacement of the normal acid-base balance of the juice in either direction, by hydrochloric acid or sodium bicarbonate instillation directly into the gut, was a rise in bicarbonate end pH of the juice followed by a prompt return to normal. During acidification and recovery, carbon dioxide tension remained within normal limits or was constant. The authors suggest that the high carbon dioxide tension may be indicative of a dual origin of jejunal juice resulting in the production of both acid and alkaline secretions. Robinson, Luckey & Mills (51) studied solutions circulated through intestinal loops in dogs and found a progressive increase in pH throughout the jejunum as a result of increased bicarbonate content and decreased carbon dioxide tension. Change of acid-base bal-

ance of the blood by administration of ammonium chloride or sodium bicarbonate did not change the carbon dioxide tension, a result similar to that noted with instillations (50). Alkalosis caused significant increases in pH, whereas the acidosis had no effect on the intestinal contents. The supposed effect of ammonium chloride acidosis in the promotion of intestinal calcium absorption is consequently denied.

*Acid-base balance and gastric secretion.*—The changes in the mineral composition or reaction of blood and other body fluids which either affect or result from gastric secretion, continue to demand the attention of investigators in this somewhat controversial field.

Kirsner, Palmer & Knowlton (52, 53), in a study of experimental and clinical hypochloremia in ten human subjects, have confirmed the results of their experiments with dogs (54). Although the severity of hypochloremia (approximately 40 mM per liter) and the alkalosis (serum pH approximately 7.70) attained in the latter were greater than those observed in man, the conclusions based on the results of both investigations were the same, namely, that by the gradual withdrawal of gastric secretion, a severe alkalosis may be induced without marked nitrogen retention, and that neither the amount, acidity, nor chloride content of the gastric juice are significantly altered by extremes of hypochloremia. These results confirmed similar work done by Nicol & Lyall (55), Hiatt (56), and others. Experimental hypochloremia induced by a sodium chloride deficient diet in man has led to similar results (57, 58, 59). Apparently, the trend of evidence indicates that gastric function occupies a unique position in chloride metabolism, in that it exhibits a marked relative stability in the face of a degree of hypochloremia which would result in the cessation or curtailment of chloride secretion or excretion in other physiological processes, such as sweating and renal excretion (54).

Studies with humans (60) showed that gastric secretory activity, or change in it, does not appreciably affect the arterial blood chloride. Mann, Grindlay & Mann (61) found that during rapid gastric secretion of acid, appreciable differences in arterial and gastric venous blood were confined mainly to decrease in the cell chlorides. On the other hand, the observation that the chloride of the red blood cells is increased at the height of gastric secretion in normal individuals (62), has been repeated in the case of patients with hyper-, hypo-, and an-acidity (63).

The venous blood carbon dioxide tension has been cited as an indicator of gastric secretion. In patients with hyperacidity, such as



an early secretory response to a test meal, an increase in venous blood carbon dioxide tension was observed, whereas in hypo- and anacidity a decrease took place (64). The authors' conclusion that a close connection exists between the carbon dioxide of the alveolar air and the secretion of gastric juice is not new, but one previously reached from observations by Dodds and others, of an increase in alveolar carbon dioxide during gastric secretion and a decrease during alkaline (pancreatic) secretion (65, 66). The view that these results are caused by acid secretion has seriously been questioned (67).

The correlation between blood carbon dioxide or alkali reserve and gastric acidity found by Apperly (68) has received little support in recent work. Kiefer (69) found that in seven of eleven cases of peptic ulcer, bicarbonate therapy resulted in unchanged plasma carbon dioxide and some decrease in gastric acid secretion. He concluded that there was little evidence that shifts in blood electrolytes, including an elevation of carbon dioxide, appreciably affect gastric secretion, except for a tendency toward decreased rate. Taylor & Michael (70) fed ammonium chloride to Pavlov pouch dogs and found no decrease in free gastric acidity, although the plasma carbon dioxide was depressed to a level one half of normal. Adams, Welch & Clark (71), on the other hand, in studies on the effect of sodium bicarbonate administered daily to Cope pouch dogs over a period of seven to eleven days found that an initial increase in gastric secretory activity in the hours immediately following a test meal was partially compensated by a decrease in later hours. The net change in gastric juice composition was an increase in free hydrochloric acid and total chloride with a decrease in total base. The daily dosage of 3 gm., consisting of three 1-gm. portions at two hour intervals, was insufficient to affect the acid-base balance noticeably, as was indicated by the fact that the changes in total chloride (decrease) and the carbon dioxide capacity (increase) of the blood were small. The results may then reasonably be ascribed to the gastric stimulating effect of small doses of bicarbonate (72) as contrasted with higher doses, which exhibit an inhibitory effect only when the amounts administered are truly excessive. The work discussed above, on hypochloremia associated with alkalosis, likewise offers evidence against the view that the acidity of the gastric juice is a rough measure of the blood bicarbonate, or that the plasma alkali reserve determines the gastric acidity.

The intimate relationship of carbon dioxide with the process of hydrochloric acid formation in the gastric glands is emphasized, how-

ever, in the carbonic anhydrase theory of gastric acid formation advocated by Davenport (73, 74) and espoused by Gray (75, 76). The latter has shown in *in vitro* experiments that carbon dioxide as bicarbonate ion is not essential for the activity of the acid-secreting cells of the stomach (76). On the other hand, since carbon dioxide as such is presumably formed in adequate quantity in the metabolism of the gland, it would seem to require no external source. Hollander (77), who discounts somewhat the significance of the evidence of arterio-venous blood chloride changes (61) from which the theory derives much of its support, has proposed an alternative scheme of membrane hydrolysis of neutral chlorides.

### MINERAL METABOLISM

*Calcium metabolism and gastric secretion.*—Calcium has been of interest in the physiology of gastric secretion from two standpoints: (a) the effect of changes in the serum calcium level on gastric secretion and acidity; and (b) the factors governing the calcium content of gastric juice itself. Babkin (78, 79) has given the most recent exposition of these problems.

The relatively few studies in the literature are in agreement that hypocalcemia results in, or is accompanied by, diminished gastric secretion and acidity. Although hypercalcemia has been more extensively studied there is less unanimity as to its effect, but the majority of studies indicate that hypercalcemia also inhibits gastric secretion. An explanation for this lack of certainty has been found by Schiffrin (80), who produced a hypercalcemia by parathyroid hormone administration in dogs with Pavlov or Heidenhain pouches. He found a great difference in the gastric secretory response of the innervated and denervated animals. A decrease in gastric volume and acidity and an increase in pepsin was shown by the former group, while an increase in volume, acidity, and chloride was shown by the latter. In both groups, hypocalcemia following parathyroidectomy resulted in an increased gastric secretory volume, little change in acidity or chloride, and a decreased pepsin concentration. Furthermore, another variable factor, namely, the amount and manner of introduction of the stimulus causing change in serum calcium level, was revealed when the injection of very small amounts of parathyroid extract decreased the serum calcium and increased the gastric secretion. In hypocalcemia the results could be reversed by intravenous calcium lactate or subcutaneous parathyroid hormone administration.

Although the concentration of calcium in gastric juice itself and the factors causing variation in the concentration have received much less attention, a similar wide divergence of results has marked our information in this respect also. Val Dez & Sendroy (81), in a study of the histamine-stimulated gastric secretion curves of normal individuals, found that gastric juice calcium was lowered at the height of acid secretion. Since their calcium values corresponded to the lowest reported in the literature, and the relationship to pH indicated that there was no calcium at all in the pure parietal secretion, they concluded that pH as a factor governing the calcium content of gastric juice is merely a reflection of the extent to which parietal hydrochloric acid secretion is diluted with nonparietal fluids or substances. These results were in accord with other observations that the parietal secretion of dogs contained no calcium (82), that mucus can be a ready source of gastric calcium (83), and that there is a reciprocal relationship between calcium and acidity in gastric juice (84, 85).

Human gastric juice has been found to contain magnesium in amounts from 0.74 to 8.8 mgm. per cent, with high values sometimes associated with a low acidity (86).

*Chemical aspects of urinary calculus formation.*—This subject has received little or no attention in past reviews, with one exception (3). While the phenomenon of stone formation is of immediate clinical importance, it is interesting in a much broader sense in that, in many respects, it appears to be closely allied to that of bone formation. The expectation that physicochemical principles could profitably be applied to this problem in mineral metabolism has stimulated many studies within recent years bearing on its etiology, prevention, and cure. The more accepted viewpoints in these respects are summarized in the monograph of Higgins (87) and in other urological reviews (88 to 95).

The following summary is based on the foregoing and other publications too numerous to permit individual citation of more than a small percentage of them. It is now apparent that the etiology of renal lithiasis is as varied as is the composition of urinary calculi, which may consist of pure or mixed solids in the form of uric acid, urates, cystine, and calcium oxalate in acid urines, and calcium oxalate, carbonate, and phosphate, and ammonium magnesium phosphate in alkaline urines (93, 96).<sup>2</sup>

<sup>2</sup> Recent examinations of urinary calculi by x-ray and optical methods indicate the complex nature of the solids formed (98, 99) and furnish information on the specific phases which have hitherto been unidentifiable by ordinary chemical analysis.

In the production of stones there is one chemical mechanism common to all, namely, the formation of crystals of one or more substances insoluble in the urine of a particular composition. Various factors may be responsible for the chemical reactions leading to the precipitation of crystals, which occurs even in normal urine (100). However, such crystallization normally results in the production of sediment, not stones. The latter apparently require some form of keratinization to produce the required framework of fibrin and mucin (87, 94, 95, 101, 102). These two essential factors, namely, urine composition and the availability of an organic matrix, represent the dual nature, chemical and pathological, of the pathogenesis of calculus formation.

The strict application of the physiochemical concept of solubility to this problem is beset, at least in the case of calcium salts, by the same difficulties cited for bone formation (2). In the absence of a solid phase particularly, urine may temporarily be "supersaturated" with respect to the concentration of ions and molecules of any of the stone-forming slightly soluble substances. Such "supersaturation" may be in part only apparent, in that it represents the modifying solvent effect of urinary solutes such as urea, the salts, and of the pH of the sample. In general, however, an excessive local concentration of the slightly soluble solutes in the urinary tract, however caused, may be expected to favor ultimate crystallization.

As causative factors in the disturbed mineral metabolism leading to renal calculus formation, the following have been cited on the basis of clinical and experimental evidence: (*a*) abnormal diet or medication (vitamin A deficiency, deficiency of calcium or magnesium, excess of calcium or phosphorus, excess of alkaline foods or, less frequently, of acid foods), (*b*) metabolic diseases (gout, hyperparathyroidism, prostatic hypertrophy), (*c*) chemotherapy (sulfa drugs, salicylates), (*d*) stasis or urethral pressure (age, pregnancy), and (*e*) urinary infection (usually associated with urea-splitting bacteria).

Vitamin A deficiency is a factor least understood, and probably the most controversial. Although the experimental production of calculi in a significant proportion of animals with avitaminosis A (103) has often been confirmed (87, 104, 105), more recent clinical investigations reveal a serious lack of agreement between those who do (101, 106 to 109) and those who do not (110, 111) find a causal relationship between vitamin A deficiency and urolithiasis in human beings. More recently, Ezickson (112) has presented additional data to the effect

that liver dysfunction (and thyroid, also) is frequently associated with this condition. This confused situation owes its origin, in part, to the indiscriminate application to clinical conditions, of experimental results with laboratory animals. Nevertheless, there is the strong indication that some one or several metabolic factors, more or less closely associated with the metabolism of vitamin A, may, under conditions not well understood, be responsible for a pathological (epithelial changes and local lesions) or perhaps a chemical setting favorable for stone formation.

The close relationship between urolithiasis and hyperparathyroidism is well established (113). However, because of its relative infrequency compared with those of other causative conditions, hyperparathyroidism accounts for much less than one per cent of all cases of urinary calculus formation (87, 114, 115).

*Dissolution of calculi in vivo.*—To combat the chemical factor, suitable dietary regimes have been designed and employed to make the reaction and composition of the patient's urine favorable to the maintenance in solution of the constituents of the particular stones involved. Thus, prophylactic therapy is greatly dependent on urine analysis and the identification of the stones found in each individual case. Recent papers present improved methods for such analyses (96, 97, 116 to 122). Although the individual variation makes impossible the institution of a control in human beings comparable to that in experiments with animals (123 to 127), clinical experience indicates a certain measure of success in following the dietetic therapy so indicated. Infections involving urea-splitting organisms such as *Bacillus proteus vulgaris* are admittedly the most difficult to treat (100, 128). The constant formation of ammonia makes almost impossible the production of a urine sufficiently acid to prevent numerous recurrences of stones consisting predominantly of calcium and phosphate.

The search (129, 130) for substances which will dissolve formed stones *in vivo* has largely been confined to work on calcium phosphate calculi. Albright and his co-workers have found that the requirements to be met by a suitable substance, namely, that it be nontoxic, a natural body metabolite, bactericidal, and sufficiently acid to dissolve calcium phosphate without increasing the excretion (more properly, the concentration) of calcium and phosphate ions in the urine, are fulfilled by citric acid (131, 132, 133). Acid citrate buffer solutions of pH 4.0 have been found to dissolve calcium phosphate stones *in vitro* and *in vivo*. Magnesium, as magnesium oxide) has been added to eliminate

bladder mucous membrane irritability and to increase the solubility of the calcium salts (134). The bactericidal action of the improved "Solution G" (135) has been reported effective in the prevention of calculi formation in urines infected with urea-splitting organisms (136).

*Citric acid metabolism and calculus formation.*—In harmony with the above but presenting a new viewpoint, are other studies which indicate the importance of a regulated citric acid metabolism in preventing calcium urolithiasis in the normal individual. Kissin & Locks (137) have found that the urinary excretion of citrate is lower in adults with calcium calculi than in normal subjects, an observation soon confirmed by others (138, 139). Shorr, *et al.* (138) have found that this excretion in normals parallels that of calcium, and varies with the calcium intake. A renal mechanism is postulated in which the citrate concentration of the urine is regulated by the calcium present and in such a manner that while the total calcium concentration is unaffected, the concentration of ionized calcium is adjusted at a level lower than that required for calcium phosphate or carbonate precipitation (140, 141, 142). In the urolithic patient this adjustment of the citrate to the extent required by the degree of calciuria fails. Further evidence that a renal factor in citrate metabolism is involved has been provided by Scott, Huggins & Selman (139), who found that less citrate was excreted in urolithiasis, as compared with normal controls, even when citrate intake was greatly increased, although other evidence indicated that the bowel absorption of citrate and its extra-renal catabolism were comparatively normal. The authors suggest that their results are at least presumptive evidence of an increased intra-renal citrate oxidation in urolithiasis. Mårtensson (143, 144) has, in fact, shown that in mammals citric acid is most vigorously oxidized in the kidney, this organ being the chief site of disposal of this metabolite. Administered citrate is metabolized mostly to bicarbonate and carbon dioxide in normals, but some of it appears as such in an increased excretion in the urine. Although the mechanism of kidney citrate excretion is still not clear, the existence of this renal metabolic factor, whether it operates by selective reabsorption, by oxidation, or otherwise, indicates the difficulty in bringing about the suggested increase (137) by citrate administration, of the urinary citrate concentration in urolithiasis.

*Citric acid and bone salt metabolism.*—Recent developments bearing on calcification and other aspects of bone physiology have been summarized in a review by McLean (2) which includes a sound and

interesting treatment of bone as a tissue and extends the coverage of previous reviewers, whose works are listed. Armstrong's timely summary (145), from a somewhat different standpoint, is concerned with the pathological aspects of bone metabolism, and treats the general metabolic sequelae of fractures and the factors which influence their healing. In this discussion, therefore, it will suffice to cover developments during the past year or so, and to refer briefly to phases not previously considered in other reviews, but which have assumed increased interest or significance with the passage of time.

A discovery, subsequently confirmed (146, 147), which may possess significance much greater than the attention it has received, is that of Dickens (148, 149) who found, in several species of common domestic and laboratory animals, as much as 70 per cent of the body content of citrate present in the hard substance of bone. This fact, which has hitherto not been observed presumably because citrate has been analyzed as carbonate, constitutes a strong link in the growing chain of evidence for the participation of citric acid in calcium metabolism. Such a development, which has a rich background in, and is a logical consequence of, chemical studies of the properties of calcium and magnesium citrates, has far-reaching implications not only with respect to bone metabolism, and especially calcification, but to the state of calcium and magnesium in the fluids of the body, and to problems of the origin of endogenous citrate in the living animal. It is in harmony with the recent evidence of the role of citrate in renal calculus formation considered in the foregoing, the use of citrate in the treatment of rickets (150), and the recognition of a small but appreciable concentration of a bound but diffusible form of calcium in body fluids (151). It should be emphasized that it is not the concentration, but the rate of movement and reaction, or the time factor in the availability of a substance which determines its quantitative significance in metabolic processes. Dickens (149) has recognized this in the suggestion that bone citrate, by a rapid turnover accompanying other bone salts such as phosphate (152), may readily be available as an endogenous source of supply. However, this expectation has not been realized experimentally. Thus, Class & Smith (153) concluded that the excess renal excretion of citrate after bicarbonate and malate feeding in rats can only be attributed to intermediary metabolic, and not to skeletal, origin. Their results were confirmed in part by Leonards & Free (154), who likewise failed to find an alteration in the citrate content of the bones of rats as a result of prolonged sodium citrate or am-

monium chloride feeding. These results mean that the stimulus required to mobilize endogenous citric acid from bone tissue is not furnished by measures which result in the urinary excretion of endogenous citric acid, preformed or synthesized, derived from other tissues (155, 156, 157). Such evidence does not exclude the possibility of the mobilization of citric acid from bone, or the deposition of it in that tissue, under other conditions favorable for the operation of some controlling factor. Nicolaysen & Nordbø (158) report that lack of vitamin D results in a decrease of bone citric acid content.

Further evidence of the important role of citric acid in bone and calcium metabolism is derived from other sources. Shohl has demonstrated the favorable effect of oral administration of citrate buffer in the prevention and cure of rickets in rats (150) on a rachitogenic diet (calcium and phosphorus intake imbalanced), and has reported success in the healing of rickets in two infants in the absence of a dietary vitamin D supplement (159). Others have studied the effect of injected citrate in rats, in the absence of vitamin D, and found that the citric acid in the bones was substantially lowered, but that citric acid by injection did not cure rickets (158). The injection of sodium citrate was followed by a marked hypocalcemia and tetany (with higher doses) in rats (160). When given by intravenous infusion in cows or by stomach tube in rabbits, sodium citrate caused no tetany, but an increased excretion, mainly renal, of calcium to the extent of about 80 and 22 per cent, respectively (161). The effects suggested mobilization of salt from the bones. A similar result was obtained by Gomori & Gulyas (162), who injected dogs (puppies) with sodium citrate. A prompt and marked increase in the urinary excretion of calcium was observed, in agreement with others (143, 144, 3, p. 137). The less marked urinary phosphate excretion was delayed. The levels of calcium and phosphate in the blood remained essentially unchanged. Histological examination of the bones of puppies and rats repeatedly injected with citrate gave evidence of a marked similarity to parathyroid hormone action; the microscopic bone changes closely resembled those occurring as a result of toxic doses of that hormone.

*Composition of bone salts.*—Additional contributions (163, 164, 165) regarding the chemical composition and crystal structure of bone minerals continue to support the differing points of view already summarized (2). Although lack of agreement as to exact composition may arise from differences in technique, interpretation, and the material studied by the investigators, it serves to strengthen the growing con-



cept that the minerals of bone have not only a structural but also an active metabolic function, hence bone possesses some degree of lability in composition of the constituent elements. Hodge *et al.* (166), using the radioactive isotope, have studied the hitherto undetermined nature of the sodium fraction of bone and other calcified tissues. These investigators found that the uptake by the tissues of this element from solution and the relationship of the amounts in bone and blood, could both be explained satisfactorily as adsorption phenomena. In the absence of more direct evidence for sodium as a replacement atom in apatite, they regard bone sodium as a constituent adsorbed on the surface of the hydroxyapatite (or, presumably, whatever form the principal molecular component of bone may take). Physiologically, this is quite consistent with other evidence indicating the labile nature of bone sodium and its possible function as a reserve in the maintenance, at some stage of depletion, of the extracellular body fluids.

*Parathyroid hormone action.*—Recent summaries of the literature on the metabolic action of the parathyroid gland are those of Pope & Aub (167) and of Campbell & Turner (168), which are principally concerned with the relationships of all endocrinological factors involved in calcium metabolism. Studies of the mechanism of parathyroid hormone action continue to appear, without, however, indicating a definite trend toward the settlement of the question of the extent to which the changes, resorption (dissolution, "decalcification") and formation of bone, and the maintenance or adjustment of the serum calcium level, depend on the urinary excretion of phosphate. That the hormone has a direct action on the kidney, resulting in an increased excretion of phosphate, is now well established despite an occasional result (169) apparently to the contrary. There is also evidence for the view that the parathyroids, or at least certain extracts of them, act directly on bone, causing histologically observable changes in its structure (170), as the mobilization of bone calcium is reflected in an increased concentration of calcium in the plasma (171). Moreover, such resorption has been likewise observed in nephrectomized animals (172, 173). More recent articles by Selye (174) and others (175) indicate clearly that the parathyroid hormone acts directly on the bones independently of any mediating action of the kidney. On the basis of such evidence, it has been acknowledged that the parathyroid hormone effects, on phosphate excretion and on bone salt mobilization, were distinct and independent (6, 171, 176). However, an opposite point of view (92) favoring the dominance or precedence of

kidney function over direct bone action has been maintained by those (173, 177, 178) who, holding to the hypercalcemic response as a criterion of parathyroid hormone action, have failed to obtain an increased serum calcium following hormone injection in nephrectomized animals.

More recently, however, a new approach from this angle has been made in two laboratories, the reported results from which are practically identical. Stoerck (179), noting several objections to the expectation that nephrectomized animals would respond to an excess of parathyroid hormone as do normal animals, studied the effects of parathyroidectomy in nephrectomized rats. He found an unchanged serum calcium resulted from nephrectomy alone, and a decrease of one-third when parathyroidectomy was superimposed. A marked increase of 100 per cent in serum phosphate resulted from both conditions. These results indicated that the normal serum calcium concentration could be maintained without the kidneys, but not without the parathyroids. The nephrectomized, parathyroidectomized animals showed, moreover, an increase in serum calcium to a normal value when injected with parathyroid hormone. In similar experiments with dogs, Monahan & Freeman (180, 181) also found that the normal serum calcium level of the nephrectomized animals was lowered about 50 per cent in the absence of the parathyroids. These experiments constitute proof that the hormone can effect an elevation of serum calcium without the intervention or co-operation of the kidneys. However, the true hypercalcemia produced under the influence of the parathyroids has yet to be evoked in nephrectomized animals. The action of the hormone under normal physiological conditions is still not settled. In all likelihood it will be found that in the several adjustments required to maintain the serum calcium level, and in the steady state characterizing bone metabolism, both direct action on the bone and on kidney function are at different times mutually or independently involved.

Other possible mechanisms or sites of action deserve consideration. The suggestion has been made that the secretion elaborated by the parathyroids may be multiple in function, perhaps consisting of more than one hormone (182). The liver has been studied as a site of parathyroid action (183, 184), more recently by Tweedy & Campbell (185, 186), who suggest a direct action of parathyroid hormone on phosphorus metabolism in the liver, based on results indicating a definite acceleration of the uptake of radioactive phosphorus by the liver following the administration of the hormone. An accelerated movement of

labeled phosphate from the femurs of treated animals is interpreted as evidence of hormone action on the phosphorus metabolism of the liver and kidneys, rather than on the bones. Radioactive strontium showed a marked accumulation in the kidneys of rats injected with parathyroid hormone (187).

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## NUTRITION

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This review will deal principally with studies relating to human nutrition. The particular papers to be mentioned are those which have come to our attention and have been of interest to us. More space has been given to those subjects which have not been reviewed recently. No attempt is made to mention all papers that have appeared this year on these subjects, as abstract journals are available for this purpose. Work dealing with the chemistry or physiology of individual nutrients or their relation to animal nutrition will not be discussed. It is to be expected that there will be a certain amount of overlapping with the reviews on vitamins, proteins, and minerals since these are the basis of nutrition. It was not thought wise to "divide" the papers with the other reviewers since the points of view and interpretations of similar data may vary. Throughout the review the Recommended Dietary Allowances of the Food and Nutrition Board, National Research Council (1), will be referred to as RDA.

### ENERGY

Nutritional surveys (2, 3, 4) suggest that the RDA for calories are somewhat too high for some groups, especially for adolescents and adults. Most dietary histories do not yield data useful for calculating caloric requirements, although carefully taken histories might be of value, since there are no data to suggest that caloric intakes above maintenance are of value. Perhaps environmental temperature should be given more consideration. Swift (5) has shown that for the rat the caloric expenditure at 15 degrees is approximately twice that at 29 to 32 degrees, the critical temperature of the rat. Furthermore, approximately two thirds of the energy released by specific dynamic action (SDA) is useful to the rat at these lower temperatures. The SDA of protein, fat, carbohydrate, and of these materials in various combinations, has also been studied with the rat (6). In these studies the rats were maintained at a constant level of nutrition, rather than fasting, which should make the studies more comparable to practical conditions. The SDA was 32 per cent for protein, 20 per cent for carbohydrate, and 16 per cent for fat. In combinations, however, the SDA

of protein and fat, and of carbohydrate and fat were lower than that of fat alone. It thus appears that fat is of more importance than protein in determining the SDA of mixed diets. Also it is clear that the SDA of the various constituents in a diet are not additive, that they must be determined experimentally for each diet, and that they will perhaps vary with the nutritive state of the animal. Slight differences observed experimentally when fat is substituted isocalorically for carbohydrate in animal diets should be carefully evaluated in the light of these data. It is of considerable interest also that with the mixture of fat, carbohydrate, and protein the SDA amounted to 18.1 per cent. These studies suggest further reasons for variance between standards and observed intakes and emphasize the need for similar studies on man.

### PROTEIN

Renewed interest in the protein requirements and the essential amino acids for man together with improved methods of amino acid analysis (7, 8) may soon lead to a redefinition of protein requirement in terms of the individual amino acids and an evaluation of diets on this basis. Block (9) has suggested allowances for the amino acids based on analyses of an "average American diet" and on calculations from the amino acid requirements of rats. Several objections can be made to such figures and in all probability they are much too high. The protein allowance of 70 gm. includes at least a 50 per cent excess to allow for proteins of low biological value. However, there is no evidence that an excess of protein over that required for nitrogen balance is of value to the adult and thus amino acid allowances might well be set at a conservative level nearer actual requirements. The young rat requires about 18 per cent of high quality protein in the ration whereas the adult rat may easily be maintained in nitrogen balance at less than one third this amount. Even with these divergent levels of protein, the food consumption of the young rat per kilo of body weight is about twice that of the adult rat. For larger animals the intake per kilo of body weight is even less.

A closer approach to actual requirements can probably be made on calculations based on the amino acid composition of high quality proteins. According to definition (10) a protein that is highly digestible and with a biological value of 100 is completely utilized and will replace the nitrogen losses occurring on a nitrogen-free diet without any excess nitrogen appearing in the urine. Thus the nitrogen excretion

on a nitrogen-free diet represents the amount of nitrogen required by the adult from such a protein. Egg protein approximates these requirements (11), and direct evidence is being obtained that it will completely replace endogenous protein losses without wasted nitrogen (12). The data compiled by Mitchell & Hamilton (13) from the older literature show losses of nitrogen on nitrogen-free diets equivalent to 0.156 to 0.38 gm. of protein per kilo of body weight per day in man. Thus 0.4 gm. of egg protein per kilo of body weight should fulfill the daily nitrogen requirement for the adult. This would supply for a 70 kilo man the amounts of amino acids shown in Table I, where they are compared with those presented by Block. The amino acid figures for egg protein are taken from the data of Block & Bolling (7) although there is evidence that the leucine figure may be too high and the valine figure too low (7). There is no assurance that such figures represent minimum values but they should be adequate. The incongruity of Block's amino acid allowances and actual requirements is evident since by his allowances 70 gm. of egg or beef protein would be inadequate (14, 15).

TABLE I

CALCULATED VALUES FOR THE AMINO ACID REQUIREMENT OF MAN (70 Kg.)  
COMPARED TO THE VALUES GIVEN BY BLOCK (9)

Amino Acid	Block, from Rat Data (gm/day)	Block, from Diet Analysis (gm/day)	Authors, from Egg Protein (gm/day)
Arginine .....	1.2	4.7	1.8
Histidine .....	2.4	2.0	0.5
Lysine .....	6.0	5.2	1.4
Tyrosine .....	...	3.9	1.0
Tryptophane .....	1.2	1.1	0.4
Phenylalanine .....	4.2	4.7	1.4
Cystine .....	3.7	4.1	{ 0.5
Methionine .....			{ 1.4
Threonine .....	3.6	3.6	1.2
Leucine .....	5.4	12.6	3.6
Isoleucine .....	3.0	3.7	1.3
Valine .....	4.2	3.9	1.3

Care must be used in applying the data obtained with one species in calculating protein requirements of another. Evidence from studies in this laboratory (16) indicates that the growing rat's requirements for certain amino acids are many times that of the adult dog whether calculated as percentage of the ration, the intake per kilo of body

weight, or as percentage of the calories consumed. It remains to be seen whether "biological values" which are dependent upon the ratio of amino acids in the protein will be the same for different species. Biological values for several proteins determined with human subjects have been reported (11). Lintzel (17) found that 34 gm. of potato protein per day maintained nitrogen balance in a 70 kg. man and that studies with meat and milk yielded surprisingly poor results. Whether Lintzel means that meat and milk were less efficient than potato or simply not superior is not clear from the abstract of his paper.

Direct studies on the essential amino acids for man have been continued. A further report on the role of methionine and cystine (18) showed that methionine was essential for nitrogen balance while cystine may be replaced by methionine. This confirms the previous report of Rose *et al.* (19). Albanese (20) has also compared the effect of the administration of tryptophane, methionine, and phenylalanine as the naturally occurring *l* form and as *dl* mixtures. It appears that the *d* forms of tryptophane and phenylalanine are less well utilized than the naturally occurring form, whereas both forms of methionine are utilized. Since these amino acids were administered singly to fasting individuals, it is not certain from these studies that the same situation would hold when the amino acids were administered with a complete amino acid mixture. Experimental data (21) suggest that the rate and time of administration may be of importance in the efficacy of amino acid utilization.

European wartime diets high in indigestible residue are reported to double or triple the normal fecal nitrogen (22). Nitrogen equivalent to 18 to 30 gm. of protein was found in the fecal excretions. Thus again there is a "vicious cycle" in which the diet becomes poor in protein and high in roughage which in turn decreases the available protein.

As with calories, temperature may be of some importance in protein requirements. Mills (23) finds the casein requirement of the rat to be increased at high temperatures. Physical fitness studies on human subjects (24), however, do not indicate that environmental temperature is of importance over short periods. No changes in physical fitness during six-week experiments at temperate and hot environments were noted at widely different levels of protein intake. In confirmation of the data mentioned above with regard to specific dynamic action, metabolic studies indicated that high protein diets were not objectionable in hot environments. In another study (25) young men

were found to show no change in physical fitness or efficiency during two-month experimental periods when fed diets containing only 50 to 55 gm. of protein, of which 90 per cent or more was from vegetable sources. High levels of protein also failed to show beneficial or harmful effects. It is of considerable interest that in these studies where nearly all of the protein was supplied by natural foods of vegetable origin, the protein intake per kilo of body weight per day varied between 0.57 and 0.85 gm., averaging 0.67 gm. The protein intake was as low as possible without resorting to the use of large amounts of nitrogen-free constituents. These data suggest that caloric restriction or very unusual diets may be needed to produce protein deficiency.

#### MINERALS

*Iron.*—In the past year data on population surveys have become available which throw some light on the human iron requirement. In any discussion of iron requirement it must be borne in mind that a number of factors in iron absorption may alter the requirement among individuals and perhaps from one population group to another. Several reports give information on the nutritional status in England with respect to iron. Davidson & Donaldson (26) observed an increase in blood hemoglobin of Edinburgh municipal schoolchildren who were given 3 to 6 grains of ferrous sulphate daily. The average changes in three treated groups were from 85.3, 80.3, and 80.7 per cent to 87.0, 85.9, and 86.3 per cent respectively. In the untreated groups the average values were 85.4, 77.9, and 82.2 per cent to 84.6, 80.1, and 82.6 per cent respectively. Additions of ascorbic acid to the iron supplement did not produce an increment in the hemoglobin level over that given by iron alone. Yudkin (27) studied the hemoglobin levels of 866 WAAF personnel and found higher average values in the regular personnel than in the inductees. Five hundred and fifty-nine inductees averaged 94.84 per cent hemoglobin while 307 WAAF personnel of over six months service averaged 103.05 per cent. The improvement was ascribed to higher iron intakes in the service. The average daily intake was estimated to be 35 mg., much of which was believed to come from iron cooking vessels used in the WAAF kitchens.

Davidson and co-workers (28) studied hemoglobin levels of 3,338 inhabitants of Edinburgh. Of 917 municipal schoolchildren, 39 per cent had hemoglobin levels of 80 per cent or below, whereas only 4.7 per cent of private schoolchildren were in this range and these were

all girls. Twenty-one and one-half per cent of female factory workers had hemoglobin levels in the range 61 to 80 per cent, and 72.3 per cent of the pregnant women studied had levels of 80 per cent or less. Male students were all above 91 per cent and only 1.6 per cent of the male factory workers were below 86 per cent.

Two other English hemoglobin surveys indicate that however suboptimal in iron the diet is, it has shown gradual improvement in recent years. Fullerton and co-workers (29) compared present day hemoglobin levels of poor people in Aberdeen with those of a similar survey in 1935. In nearly all of the school age groups, the 1943 hemoglobin averages were higher, as was true of the pregnant women and adolescent and adult males studied. Adolescent girls engaged in industry showed the opposite trend. The general trend toward better hemoglobin values was tentatively explained by the use of the national wheat bread which increases the per capita iron intake. Davidson and co-workers (30) compared the hemoglobin levels of schoolchildren and pregnant women of Edinburgh in the years 1942 to 1943 with those found in 1944. Average hemoglobin values for schoolchildren in two schools for the summer of 1942 were 82.9 and 77.5 per cent respectively. These had increased to 87.5 and 90.8 per cent by June, 1944. Average values for pregnant women in the second trimester were 77.0 and 86.8 per cent for these two years. Again the authors attribute this improvement to use of the national wheat meal flour. Using the value of 0.66 mg. of iron per ounce of bread, the authors estimated an average daily iron intake of 19 mg. for Edinburgh children, which represents an increase of 5 mg. over the estimated 14 mg. intake when white bread was used. If these estimates are correct, it would appear that under these conditions optimal hemoglobin values will not be reached in schoolchildren receiving 14 mg. of iron.

In view of higher American hemoglobin standards and considering the response of some of these groups to iron supplements, it seems clear that the English diet is suboptimal in iron. Whether this situation implies a hazard to health is unknown.

Milam & Anderson (2), from a nutrition survey of a rural county in North Carolina, estimated that the average daily iron intake of 457 whites and 399 negroes was 9.7 mg. and 8.3 mg. respectively. Average hemoglobin values in children under twelve years of age were 13.0 gm. per cent for both white boys and girls and 12.3 and 12.4 for negro boys and girls. Average values of all persons over twelve years of age were 14.6 and 13.1 gm. per cent for white males and females and

14.1 and 12.5 for negro males and females. The daily iron intakes recorded in the North Carolina studies are slightly lower than the 10.3 mg. average estimated for Oklahoma college women (31). These figures indicate somewhat better hemoglobin values on lower iron intakes than have been observed in England.

*Fluorine.*—Interest in fluorine and its relation to dental health has stimulated considerable epidemiological study of the effects of various quantities of water-borne fluorides on the population. The maximum concentration of fluorine permissible in drinking water is 1.0 p.p.m. according to the Public Health Service Drinking Water Standards (32). Considerable evidence indicates that this concentration appears to be about optimal for protection against human dental caries and yet is not high enough to induce dental fluorosis (33). In England, Weaver (34) found that the incidence of dental caries in children in a town with a water supply containing 1.5 p.p.m. fluorine was only 56 per cent of that found in children in a neighboring town with a water supply containing 0.25 p.p.m. fluorine. The difference in caries incidence was greater for incisor than for molar teeth. An interesting study in public health has been set up in New York State in the cities of Newburgh and Kingston. The water supply of Newburgh is to be fluorinated to a content of about 1 p.p.m., whereas that of Kingston will remain essentially fluorine free. All 5 to 14 year old children in the schools of the two cities are to be given annual dental examinations and representative groups will be given periodic physical examinations. Controlled epidemiological studies of this type will be of great value in guiding future public health activity toward mass improvement in dental health.

Possible harmful effects of consumption of drinking waters containing fluorine have been investigated by the U.S. Public Health Service. McClure (35) studied the relation between fluorine ingestion (from drinking waters) and the height, body weight, and bone fracture experience of selected groups of 1,458 high school boys and 2,529 young adult men taking the physical examination at induction centers. The subjects were from areas in which the water-borne fluoride content ranged from 0.0 to 6.0 p.p.m. No relation was found between fluoride intake and incidence of bone fractures nor was there any relation between height-weight figures and fluoride intake.

In another report McClure (36) studied the relation between the concentration of fluorine in the water supply and that in the urine of subjects drinking the water. Fluorine analyses were done on urine



samples from over 1,900 men and boys from areas in which the drinking water varied from 0.0 to 5.8 p.p.m. fluorine. These studies show that where domestic waters are free of fluorine, the urinary fluorine content is 0.3 to 0.5 p.p.m. When the domestic waters contain as little as 0.5 p.p.m., detectable increases in urinary fluorine are observed, and further increase in the fluorine content of the waters produces proportional increases in urinary fluorine. These results show a remarkable ability of the body to handle water-borne fluorides in the concentrations encountered in these studies. They also indicate that the food fluorine intake is quite constant regardless of the locality, and amounts to about 0.3 to 0.5 mg. daily. It would appear, therefore, that with respect to fluorine the water-borne fluorides are of first importance in maintenance of dental health.

Arnold (37) proposes the controlled addition of fluorine to drinking waters in quantities not to exceed 1.0 p.p.m. In a review of benefits calculated from epidemiological surveys he states that such addition would result in a sixfold increase in the number of children with no carious teeth, a 60 per cent lower dental caries experience rate, a 75 per cent decrease in the first permanent molar loss, and about a 95 per cent decrease in caries in the proximal surfaces of the four upper incisors. McClure (38) states that 0.5 to 1.0 mg. of water-borne fluorides should be consumed daily by children of one to eight years of age and suggests that communities consider fluorinating drinking waters since evidence indicates no health hazard from these quantities. This point of view will undoubtedly come to the fore as caution peculiar to fluorine is gradually overcome.

*Others.*—McKay and co-workers (39) studied calcium retention in college women on "well-selected diets" and found no significant effects of vitamin D on the retention of calcium. Daily calcium retentions were 0.1 gm. or less as might be expected in women of this age. Kraut & Wecker (40) in studies of two months' duration in two 20 year old women found calcium balances of  $+0.01$  and  $-0.015$  gm. on intakes of 0.5 gm. calcium per day.

Johnston (41) found retention of 4 to 14 mg. per kg. of calcium in nine to fifteen year old girls receiving 21 to 35 mg. per kg. of dietary calcium per day without supplementary vitamin D. In a case of slipped femoral epiphysis calcium retention was increased from 175 mg. to 368 mg. by supplementing the diet with 1,950 units of vitamin D. The requirements for calcium and vitamin D to meet the rapid skeletal growth of this prepuberty growth are stressed in this report.

Leverton & Binkley (42) studied the copper retention of college women on self-chosen diets and on a constant diet. The average retention on the former was 0.85 mg. (32 per cent of the intake) and on the latter 0.23 mg. (11 per cent of the intake). Higher retentions of copper were observed on higher intakes. Macy (43) summarized the average daily retention of eleven essential minerals in five, eight, and eleven year old children.

### VITAMINS

*Vitamins A and D.*—Batchelder & Ebbs (44) studied the dark adaptation in four young adults over a 200 day period. The subjects were fed a vitamin A-low diet or diets containing known amounts of vitamin A. Individual variation was great and one subject failed to show an increase in adaptation time although receiving the deficient diet at all times. Their data are interpreted to mean that from 4,000 to 5,000 I.U. per day are required to maintain dark adaptation, whether at a normal level or at some point above normal. Presumably much larger amounts are needed to bring depleted individuals back to a normal range of adaptation. It is of interest that one subject showing poor dark adaptation nevertheless had normal plasma levels of vitamin A and carotene.

Many studies [reviewed by Popper (45) and Spector, McKhann & Meserve (46)] have shown a drop in blood level of vitamin A during various types of disease. Recent studies show a similar drop in rheumatic fever (47). Popper suggests from fluorescent microscopy studies that this may be explainable on the basis of a shift of vitamin A from normal to pathological sites in the liver, since total liver stores are not depleted. In normal experimental animals (45) blood levels show good correlation with liver reserves. Thus it is interesting to speculate on whether such drops in the plasma level may represent an actual "functional avitaminosis" even though liver stores are adequate. Popper states that large doses of vitamin A will cause storage of the vitamin in normal sites, but it remains to be shown whether this finding is of value in the treatment of these conditions. One report (48) states that patients with cirrhosis showed a nyctalopia which did not respond to vitamin A administration. Meanwhile, caution must be used in the interpretation of plasma levels as an indication of vitamin A deficiency. This is especially true of plasma values in ill people.

The studies of Follis and associates (49) on the occurrence of rickets in children are interesting. By histological examination, 46.5

per cent of 230 children between the ages of two and fourteen who died from all causes showed evidence of rickets. Twenty-three per cent were classified as slight, 18.7 as moderate, and 4.8 as severe. In contrast to these figures, only five cases were recognized by Roentgen examination. Although the studies were made on a hospital population, approximately 50 per cent of the children died within fourteen days after admission, and the authors felt that the degree of rickets was greater than could have developed in this time. Thus the study suggests a frequent occurrence in healthy-appearing children. No definite relationship between rickets and any other disease was established although children dying of acute disease showed a greater incidence than those dying of chronic disease. We do not feel qualified to judge the histological data these workers present as evidence of rickets nor the significance of their findings.

*Vitamin C.*—Numerous criteria have been used in attempts to establish the human requirement of vitamin C. The amount required to produce or maintain "saturation" as established by various procedures has received considerable study. It has again been shown (50 to 54) that amounts of 1 mg. per kg. of body weight per day, or slightly more, are needed to maintain "saturation." These amounts are comparable to the RDA for ascorbic acid. In the past year or so, evidence has accumulated to show that the requirement of ascorbic acid for "saturation" is considerably greater than that necessary for the maintenance of health. In a survey of 546 high school pupils in a suburb of Toronto, Riggs and co-workers (55) found that 75 per cent of the girls and 80 per cent of the boys received less than 70 per cent of the RDA for vitamin C. Medical examination revealed no impairment in the health of these children nor evidence that they had suffered from this lower intake. Perhaps the most forcible arguments come from England where the population has subsisted for over five years on vitamin C intakes estimated to be about 20 mg. per day (56), without evidence of widespread vitamin C deficiency, and where numerous studies have shown no improvement following daily supplementation with vitamin C. Indeed there is considerable evidence that the over-all nutrition of the English people has improved during the war period. In a study of Cambridge schoolchildren Yudkin (57) found that feeding vitamin pills containing 25 mg. of vitamin C to half the children for one year produced no favorable effect, as compared with control children not receiving the pills. Observations were made on growth (weight and height), blood hemoglobin, strength of

grip, dark adaptation, resting pulse rate, vital capacity, breath holding time, and endurance as measured by the RAF mercury test. There was, of course, an improvement in vitamin C saturation. Bransby and co-workers (58) in a study of 1,242 school children of Ipswich, Glossop, and London found that six months' feeding of vitamin capsules containing 20 mg. ascorbic acid produced no significant effect on rate of growth, "nutritional status," muscular strength, condition of the teeth and gums, or absence from school on account of illness. In a study of 214 adult men engaged in hard physical labor, no significant effects on weight, blood hemoglobin, blood pressure, absence from illness, or output of material resulted from the feeding of these same capsules. Asher (59) found no greater weight gain in schoolchildren receiving a vitamin and mineral supplement containing 20 mg. ascorbic acid than in those receiving no supplement.

Stamm, Macrae & Yudkin (60) studied the incidence of bleeding gums in RAF personnel and reported no greater improvement in the vitamin C fed groups (200 mg. ascorbic acid daily for one week and 100 mg. daily for the following two weeks) than in the controls. They found that the average daily intake of ascorbic acid per person at three RAF stations was 25.8 mg. in October and November, 1941, and 16.8 mg. in March, 1942. This confirms other reports as to the low level of vitamin C in the English diet. It is of interest that neither "sponginess" nor bleeding of the gums was improved by ascorbic acid. The gum conditions were found to be variable in the degree of involvement, and there was considerable spontaneous remission and reappearance in individuals. Day & Shourie (61) found no decrease in gingivitis in East Indian children after feeding 100 mg. of ascorbic acid daily for 100 days. Crandon, Lund & Dill (62), in experimental human scurvy, found that the gums did not become swollen and hemorrhagic before other signs of scurvy appeared. Farmer (63) in another study of experimental scurvy in man confirms this point, and McNee & Reid (64) found patients with sore bleeding gums to be no more "unsaturated" with respect to vitamin C than others with normal gums.

Kylos and co-workers (65) report that daily supplements of 75 to 100 mg. ascorbic acid were necessary to maintain plasma levels of 0.8 mg. per cent in a group of male prisoners and that oral examinations revealed lesions of the gums which were thought to respond to vitamin C therapy. The authors conclude as follows: "The probable minimal daily amount of vitamin C needed by healthy male adults lies in the neighborhood of 75 mg., as gauged by the response of the

plasma ascorbic acid values and health of the gum tissues." This statement is contrary to much data gathered in nutrition surveys and to practical experience. It is known that many individuals have normal appearing gums on plasma levels of ascorbic acid which are less than one fourth of the "borderline 0.8 level." The authors recognize that disease of the gums may be caused by factors other than insufficient ascorbic acid, and, because the gums are so frequently exposed to various types of minor trauma, this is probably true in the majority of cases. As has been noted, gum changes are difficult to evaluate objectively, particularly in adults who have "lost more than a third of their teeth" or are edentulous. "Slight puffiness," "occasional cyanosis," and "trend to a normal pink color" are vague signs to accept as objective evidence, and in any event are best made by a trained individual not aware of the experimental plan, for only in this way can subjective influences on the findings be reduced to a minimum. In addition, the individuals studied were not "healthy" with respect to their vitamin C status for they had all been on practically a scorbutogenic diet (not more than a "few milligrams of vitamin C" per day) for a period of one year and many for a longer time. Subjects who had been depleted of ascorbic acid as thoroughly as these may require larger amounts of ascorbic acid over long periods of time to build back tissue concentrations and hence it is doubted that the results represent the "minimum daily amount of vitamin C needed by healthy male adults." This criticism is supported by the observation that those individuals who had the longest period on the prison diet also had the lowest plasma values, more severe gum changes, and required a longer time and larger therapy for improvement. The study emphasizes the inadequate diets undoubtedly prevalent in similar institutions throughout the country.

*Thiamine, riboflavin, and nicotinic acid.*—The reader is referred to excellent reviews on the available data relating to human requirements of thiamine (66), riboflavin (67), and nicotinic acid (68). Little can be added to these summaries. Ferrebee and co-workers (69) have shown that the thiamine content of muscle decreases during thiamine depletion. However, this method of evaluating "subclinical deficiencies" ["under-the-bed" deficiencies according to Albright (70)] appears to have the same disadvantages as the saturation tests used in connection with vitamin C studies. Conclusions must be reserved until thiamine levels are correlated with symptomatic and objective findings. Perhaps a fall in intracellular thiamine would be more con-

vincing to most investigators as evidence of deficiency than a fall in the plasma level. The review on thiamine concludes that the minimum thiamine requirement of adults is probably between 0.13 and 0.17 mg. per 1000 calories and that from 0.24 to 0.44 appears protective from thiamine deficiency. A review of the same data by Melnick (71) leads him to the conclusion that 0.35 mg. per 1000 calories is near the minimum requirement. However, it is doubtful whether such figures should be set by an average of widely divergent results; rather, critical attention should be given for the differences in such conclusions. The review by Holt (66) is valuable in this respect. It is probably safe at this time to conclude that the Recommended Dietary Allowances (RDA) of the Food and Nutrition Board may well be revised downward, but the allowance to be made for "safety" will depend upon opinions which will no doubt differ.

Similarly with riboflavin and nicotinic acid there will probably be a tendency to revise the RDA downward. In neither case is there sufficient evidence to set a minimum figure, but the difficulty in meeting riboflavin allowances even on "good" diets as consumed by the U.S. Army (72) is some reason for believing they are high. Recent analyses (73) for riboflavin excretion show that many individuals excrete less than 0.3  $\mu$ g. per cc. of urine. However, this can hardly justify the authors' conclusion that "there is widespread incidence of riboflavin deficiency in this area." Williams and associates (74) found no clinical symptoms developed during a 288 day period in which only 0.35 mg. of riboflavin per 1000 calories was fed. Keys *et al.* (75) also found no adverse effects when a diet supplying 0.31 mg. per 1000 calories was eaten for a five months' period.

Nicotinic acid allowances have been based on animal experiments and dietary analyses. Valid objections can be made to both methods, and direct studies are obviously needed. Dann (68) concludes that although there is insufficient evidence to justify new figures for allowances of this vitamin, the evidence available suggests that they may be high. Reference is made to the work of Aykroyd & Swaminathan (76) showing that rice eaters rarely show pellagra although their nicotinic acid intake approximates 5 mg. per day; maize eaters, on the other hand, may develop pellagra on nicotinic acid intakes considerably above this figure. Thus the early hypothesis of a pellagragenic effect of maize (77) may still be attractive and is supported by some experimental evidence (78, 79).

Although considerable disagreement is noted in the various reports

designed to determine minimum requirements of the essential nutrients, mature consideration might lead us to expect these results. In experimental nutrition with highly standardized inbred rats, not insignificant variations are noted in the time for the onset of deficiency symptoms, and the manifestations of the disease. Much greater variation is seen in studies using the chick and dog, although the background of the animals may be known with greater certainty than is ever possible with the human being. Furthermore, most experimental nutrition is based on young growing animals well known to be more susceptible to deficiency disease and less variable than adults. On the other hand, practically all studies on the human species have been conducted with adults with no knowledge of their nutritional or genetic background, and for relatively short periods of time. Synthesis of nutrients by the intestinal flora undoubtedly affects man's dietary requirement, as shown by Najjar and associates for thiamine (80) and for riboflavin (81), and may be influenced by diet and disease. Elvehjem (82) and others have pointed to these facts and Mitchell (83), in a worthwhile discussion of adaptation to undernutrition, gives examples which may be interpreted as evidence of adaptation to changes in caloric intake and to low intakes of protein, calcium, thiamine, and vitamin A. Mitchell rejects the hypothesis (84) of "cumulative effects" of slight deficiencies as contrary to the facts. More consideration of the possibility of adaptation is deserved.

#### FOOD COMPOSITION

The Committee on Food Composition of the Food and Nutrition Board of the National Research Council has integrated a number of studies in different laboratories on analytical methods, analysis of foods (including dehydrated), and losses of nutrients during cooking. The results thus far available have been presented in two mimeographed reports entitled "Tables of Food Composition Giving Proximate Mineral and Vitamin Components of Foods" and "Vitamin Losses in Cooking Foods" (85). An extensive investigation into the nutritive value of canned foods has been organized (86). The content of ascorbic acid and carotene (87), thiamine and nicotinic acid (88), and riboflavin and pantothenic acid (89) are reported for 32 different products. The distribution between solid and liquid portions of canned vegetables and fruits of this series with respect to ascorbic acid, thiamine, and riboflavin are given (90) as well as the effects on these three vitamins of large and small scale preparation (91, 92). Cheldelin &

Williams (93) have presented tables of the riboflavin, nicotinic acid, and pantothenic acid content of foods. They estimate the "average American diet" before the use of enriched flour contained 1.4 mg. riboflavin, 11 mg. nicotinic acid, and 4.9 mg. pantothenic acid per 2500 calories. The enrichment program is believed to have increased the consumption of riboflavin by 12 per cent and nicotinic acid by 53 per cent.

The merits of enriched bread versus whole wheat bread have been extensively reviewed and discussed by Lepkovsky (94). He concludes that, "if white flour is used, even if 'enriched,' it is difficult to see how deterioration of the national diet can be avoided." This is certainly an extreme view and while the nutritional properties of whole wheat bread are superior to those of enriched white bread, from a practical viewpoint food habits must be recognized. The vast majority of Americans prefer white bread and hence the enrichment of white flour is a definite and positive step in the direction of improved dietaries, although it is less improvement than would be obtained if whole wheat bread were consumed.

#### NUTRITION IN MEDICINE AND PUBLIC HEALTH

One of the interesting developments in nutrition of the last few years has been the increased interest of the medical profession in the application of nutritional knowledge to problems of disease. Not alone in applied nutrition but also in nutrition research has the medical profession contributed. This indeed is a good trend and one that will no doubt continue to increase with resultant improvement in understanding and treatment of various diseases.

Observations of a number of workers (95) have suggested that nutrition is an important nonspecific factor in resistance to bacterial infection. The diets used in most of these experiments did not permit definite conclusions regarding the effect of any specific nutritional factors. Wooley & Sebrell (96) have shown that mice specifically deficient in either thiamine or riboflavin succumbed more rapidly to the intraperitoneal injection of virulent pneumococci. West *et al.* (97) reported observations demonstrating that albino rats deficient in pantothenic acid are less susceptible to infection with type I pneumococci than animals receiving this vitamin in the diet. The role of riboflavin, thiamine, pyridoxine, and pantothenic acid on the resistance of rats to induced pneumococcal lobar pneumonia has been studied by Rob-



inson & Siegel (98). They also considered the factor of inanition. Infection was induced with a strain of *Diplococcus pneumoniae* type I by inoculation with mucin into the trachea after thirteen to sixteen weeks on the deficient diets. The data indicate that the infection progressed with greater rapidity and produced a higher incidence of mortality in rats fed the adequate stock diet than in rats on any of the deficient diets. Seeking an explanation for the lowered incidence of induced infection in deficient animals, they measured the riboflavin, pantothenic acid, and thiamine content of the livers of the deficient animals. There was sufficient amount of these vitamins to support good growth *in vitro* of *L. casei*. On these data the authors make the assumption that vitamin deficiency of the host's tissues to the point where they will not support the growth of the invading bacteria is probably not the explanation for the resistance to infection shown by the deficient animals. This is probably correct; however, it is doubtful if the evidence presented proves the point, for it is likely that the nutritional requirements of type I pneumococcus growing *in vivo* are considerably different from those of *L. casei* *in vitro*, and the vitamin content of the liver is probably much higher than that of the infected lung and lymphoid tissue.

In protozoan infections Seeler, Ott & Gundel (99) have confirmed the observations of Trager (100) that biotin deficiency increased the severity of avian infection with *P. lophurae*. The former workers used chicks whereas Trager used the duck. In continuing this study Seeler & Ott (101) have found that riboflavin deficiency produced an opposite effect. Parasite counts were decreased in riboflavin deficient chicks and the administration of riboflavin to deficient birds during the course of the disease increased the severity of the infection. The addition of an excess amount of riboflavin to a diet already adequate in this nutrient had no effect on the infection.

The relation of nutrition to resistance to poliomyelitis virus has received the attention of several investigators. Rasmussen *et al.* (102) have found that mice fed diets deficient in thiamine show a lower incidence of infection to Theiler's virus and to Lansing strain of poliomyelitis virus than do animals fed a similar diet with optimum thiamine. In some instances the thiamine deficient mice which survived became paralyzed when given adequate thiamine. Diets restricted in caloric value but adequate in all vitamins likewise produced a decreased susceptibility but it was less marked than in the thiamine deficient mice. Foster *et al.* (103) reported similar observations, and in

a further report (104) they used the paired feeding technic to determine if an insufficient supply of thiamine, aside from its effect on food intake, exerts a direct influence on the development of the poliomyelitic infection. The authors believe that the effect of thiamine deficiency on the action of the virus is not due solely to the resulting anorexia, though the differences between the thiamine deficient group and the paired fed group were slight except for a short period approximately seventeen days after inoculation. Both groups of animals had fewer cases of paralysis and the number of deaths was less than in a control group on an unrestricted amount of the complete diet.

Aycock & Lutman (105) have prepared a review on vitamin deficiency as an epidemiologic principle. They conclude that vitamin deficiency is not a general epidemiologic principle in susceptibility to infection. They suggest that deficiencies of certain vitamins affect susceptibility to certain types of infections only in limited areas where vitamin deficiencies reach a sufficiently severe degree to produce tissue changes which are favorable sites for secondary infection.

Cirrhosis of the liver with hepatic insufficiency is a common disease of unknown etiology and one that has been rather hopeless from the viewpoint of therapy. From ancient times diet has been linked with this disease. The newer ideas relating nutrition and this disease have been discussed by Hoagland (106), and in general they stress the importance of protein, available methyl groups, and various factors of the vitamin B-complex, in contrast to the older ideas of diets high in carbohydrate and low in protein, fat, and B-complex vitamins. Patek & Post (107) have shown the clinical importance of a diet rich in protein and B-complex vitamins in the treatment of this disease. It is known that choline deficiency leads to the development of fatty livers in several species of animals. Its place in human nutrition has not been definitely established but beneficial effects have been attributed to choline in the treatment of human liver cirrhosis, and at present it is being used by several investigators in the therapy of cirrhosis of the liver as well as in various types of hepatitis. Broun & Muether (108) reported favorable results in the use of choline in the treatment of several patients with cirrhosis of the liver, although Yater (109) in a report dealing with fifteen patients observed no significant benefit from choline administration. Russakoff & Blumberg (110) described definite improvement as evidenced by clinical and laboratory findings in seven of nine patients given supplements of choline in addition to a diet high in protein, carbohydrate, B-complex vitamins, and low in

fat. Enlarged livers were reduced in size, ascites was greatly diminished or disappeared, serum proteins increased, the hemogram improved, and prothrombin time and liver function tests became normal. Three of the patients who showed little change when treated for three weeks with the high protein diet alone, showed distinct improvement within the first ten days of choline therapy.

McKibbin, Thayer & Stare (111) have described the experimental production of acute choline deficiency in weanling puppies and observed the effect of such a deficiency on several of the tests available in clinical laboratories. They found a rise in blood plasma phosphatase and prothrombin time, an impairment in bromsulfalein elimination, and a fall in blood plasma cholesterol and cholesterol esters. Another experimental study in dogs which will be of interest to clinicians is the report of Goodell, Hanson & Hawkins (112) on the susceptibility of protein depleted animals to liver injury produced by injection of mapharsen and a protective effect by methionine. Mapharsen was chosen because it is the arsenical commonly used in the treatment of syphilis. The dose generally used in man is 0.001 gm. per kg.; the dogs were given amounts varying from 0.0025 to 0.0045 gm. per kg. Adult dogs were rendered protein deficient by feeding a diet adequate in other nutrients but low in protein. In a few animals plasmaphoresis was employed to effect a more rapid protein depletion. After five to ten weeks on the low protein diet, the mapharsen was given. It was found that animals on the low protein diet were far more susceptible to the toxic effects of mapharsen than control animals on normal diets, and that methionine given the day prior to the administration of mapharsen produced considerable protection. Although the number of animals used was small, the data are interesting and help provide a basis for the use of methionine in protecting man from toxic effects of various substances injurious to the liver. The use of methionine with success in carbon tetrachloride poisoning in man has already been described in a case where one would have suspected death to have occurred (113). In this instance an aviator who accidentally consumed a large amount of carbon tetrachloride and developed severe liver damage made a surprisingly rapid recovery following treatment with methionine.

In many of the research problems stressed as a result of the war, nutrition has had an important consideration, particularly in relation to convalescence and rehabilitation, infection, and post-traumatic nitrogen loss. While reports of much of this work have been available

to certain investigators, very little of it has as yet been published. The Committee on Convalescence and Rehabilitation of the National Research Council has published an interesting report (114) in which the large losses of nitrogen and general weight loss following serious trauma and various diseases are stressed. The source of this lost protein, the mechanism of the catabolism, and ways of preventing or decreasing it have been matters of considerable and timely interest. The report emphasizes the necessity of high protein intake as part of general therapy and of beginning it promptly. The importance of good nutrition from the viewpoint of preventive medicine is emphasized. Optimum nutrition appears to be an important aspect in the general treatment of convalescence and rehabilitation.

Co Tui and associates have reported metabolic studies on patients following trauma due to burns (115) and trauma due to various types of surgery (116, 117). Comparison of nitrogen intake with total fecal and urinary nitrogen indicated a favorable nitrogen balance in the burned patients studied, but this may have been misleading because nitrogen loss via wound exudates was not included. Oral administration of large amounts of a casein digest greatly improved their patients. The authors believe that a casein digest is better tolerated and utilized than natural protein food though little data are given to support this statement. In patients convalescing from gastrectomy, marked advantages are ascribed to those who were fed a diet high in amino acids and calories. These patients were tube fed, the tube having been introduced into the stomach the night before operation. During the operation it was drawn distal to the anastomosis directly into the jejunum. In "control patients" undergoing the same type of surgery but receiving the usual type of postoperative diet there was a consistent nitrogen deficit and a prolonged period of convalescence; whereas in the better fed group there was a nitrogen surplus, a gain in weight, and a shorter period of convalescence. Objective ergography showed that postoperative asthenia disappeared in the better fed group at five to six days following surgery whereas in the other group it required more than twice as long. The plasma protein concentration of neither group showed any consistent variation. It is of interest that the well fed patients soon achieved nitrogen balance and a consistent gain in weight. As the authors point out, this is not in agreement with other work but the previous state of undernutrition of the patients may reconcile this divergence. Howard and associates (118, 119) have reported a detailed investigation on post-traumatic nitro-

gen loss in patients who had had fractures of the large bones of the lower extremities. The patients were males, otherwise healthy, and their fractures healed satisfactorily. They were all at bed rest in casts throughout the entire period of observation, and received special dietary service so that a calculation of nutrients consumed was possible. The six patients studied showed losses of body protein averaging 1,400 gm. throughout the period following fracture. This loss reached a peak on an average of 5.6 days after injury, and continued, but with a gradual decline, for an average of 35 days. It is concluded that disease atrophy, bed rest, anesthesia, fever, infection, or the use of sulfonamide compounds do not account for the major part of the nitrogen losses under discussion. In observations on the influence of the diet, it was found that at the height of the protein catabolic period the ingestion of high protein and caloric diets did not exert any appreciable sparing effect on the losses of nitrogen from the body. Another patient who was cachectic at the time he sustained multiple fractures showed no increase in protein catabolism. It has been reported that nitrogen excretion does not rise in injured rats after a period of days on a protein free diet (120) but in normally fed rats similar injury is followed by considerable losses of body nitrogen (121). It is possible that the protein catabolic process occurring in healthy individuals after trauma is a useful and normal physiological response.

Spies and associates (122, 123, 124) have presented several progress reports on the results of applying the diagnostic and therapeutic practices of the Nutrition Clinic, Hillman Hospital, Birmingham, Alabama, to the problems of rehabilitation and convalescence. They emphasize the protracted nature of dietary therapy in rehabilitation. Once the crisis of illness is past and the progress of improvement is slow, the interest of the patient wanes and the physician must persist in guiding him over the tedious days until recovery is complete. Any relaxation in this continual attention to proper nutrition may result in relapse.

It is of interest that certain nutrients have been found to have influence on the clinical course of shock in experimental animals and also in man. The successful treatment of wound shock with blood plasma or albumin is widely recognized as one of the great medical victories of recent years, though as has been emphasized this therapy has not completely solved the problem. Rosenthal and co-workers (125, 126, 127) experimentally produced shock in mice by scalding, hemorrhage, and by means of rubber band tourniquets applied to both

thighs. Their outstanding finding was the great effectiveness of sodium salts in reducing mortality to shock. Sodium chloride, acetate, succinate, bicarbonate, and lactate were equally effective. Isotonic solutions of sodium chloride appeared to be superior to hypertonic solutions and oral administration as effective as or superior to intraperitoneal or intravenous injection. The finding that mouse serum given intravenously was not superior to an equivalent amount of normal saline by mouth was "shocking." Fox (128) has reported the successful clinical trial of oral sodium lactate in the treatment of burn-shock and concludes that the results were so successful that they warrant further extensive trial of this therapy. Prinzmetal and associates (129, 130) studying shock due to scalding have confirmed the findings of Rosenthal and in addition found that certain liver extracts given prophylactically improved the action of the saline. This effect of liver extract has been corroborated by Haterius & Glassco (131).

Govier & Greer (132) have studied the effect of thiamine on the survival time of dogs with shock induced by hemorrhage and found that the thiamine treated dogs lived longer than did the controls. This observation led Govier and associates to consider whether the apparent benefit from the administration of thiamine was through its action as cocarboxylase. Blood pyruvic acid levels were high in animals in shock. In a further study (133) it was found that dogs with a low plasma level of thiamine were more susceptible to shock than animals with a high plasma level. As an explanation for these observations Govier considered the possibility that the animals' own tissue thiamine became ineffective. It had previously been shown by Ochoa (134) that under anaerobic conditions *in vitro* breakdown of cocarboxylase may occur, probably by means of a phosphatase. Grieg & Govier (135) have confirmed these observations. They determined cocarboxylase and total thiamine in skeletal muscle, liver, and duodenum of dogs before and after shock and after thiamine therapy. Shock resulted in dephosphorylation of cocarboxylase in 42 per cent of the muscle samples, 69 per cent of the duodenum samples, and 46 per cent of the liver samples. The magnitude of the dephosphorylation was variable. They also found that thiamine therapy in shock resulted in a resynthesis of cocarboxylase. Alexander (136) has reported that free thiamine of muscle rises markedly during hemorrhagic shock and that this change occurs at the expense of the cocarboxylase. Govier (137) has summarized much of the work on thiamine in relation to shock. As riboflavin and nicotinic acid are both

nutrients which are necessary in certain enzyme reactions important in cellular metabolism, it would be of interest to know their status in conditions of shock.

Addisonian pernicious anemia is a disease due to a deficiency of substance necessary for the production of normal red blood cells. This substance, which has not been identified, is normally stored in the liver of man and animals. Treatment with liver or liver extract stops the progress of the disease, improves most symptoms, and returns the blood picture to normal. The disease has been of interest to nutritionists as well as clinicians, particularly because liver which is rich in so many nutrients is effective in the cure. Castle, who has long been associated with studies of this disease, has presented an interesting report (138) in which no effect was obtained when all the known B-vitamins were used in combination with vitamin-free casein and normal human gastric juice in the therapy of this disease in ten patients. The "vitamin-free" casein, 50 gm. per day, was given "on the theoretical basis that one or more of the vitamins, even if not effective independently, might become active as a prosthetic group on the casein molecule as a result of action of gastric juice." It had previously been shown that crude casein contains extrinsic factor which is extracted with difficulty. The following nutritional factors were tested over a ten-day period and found to give no response; the figures in parentheses represent dosage per day in grams: thiamine (0.1); riboflavin (0.025 to 0.1); nicotinamide (0.1 to 0.2); pyridoxine hydrochloride (0.1); *d*-calcium pantothenate (0.1); para-aminobenzoic acid (2.0); choline chloride (0.3); *i*-inositol (0.2); biotin (0.002); xanthopterin (0.009); and "folic acid" (0.0036). The same patients who failed to respond to this treatment throughout a ten day period all responded when given beef muscle or crude casein with gastric juice. These experiments seem to show that none of the known factors of the vitamin B-complex is related in a direct simple way to the extrinsic factor. Castle and associates point out "that, nevertheless, it is reasonable to continue to regard the extrinsic factor as a thermostable component of the vitamin B-complex as yet unidentified."

Tuberculosis, like all wasting diseases, is one in which faulty nutrition is thought to predispose and good nutrition to prevent or help cure. In recent years a number of investigators have been interested in the vitamin status of tubercular patients as revealed by various blood and urine tests and dietary and therapeutic studies. Because a method was available for ascorbic acid and because this vitamin is concerned

with the formation of connective tissue, it was one of the first nutrients investigated in patients with tuberculosis. Sweany and co-workers (139) made an extensive study of vitamin C economy in 300 patients and found that in the more advanced cases there were varying degrees of depletion which were proportional to the severity of the disease and the intake of the vitamin. Their patients were improved by administration of the vitamin, and Sweany *et al.* state that adequate vitamin C is one of the many factors necessary for the proper treatment of tuberculosis. Getz and associates (140, 141) have reported studies on blood levels of ascorbic acid, vitamin A, carotene, calcium, phosphorus, phosphatase, hemoglobin, and serum albumin and globulin. They found that subjects with far advanced tuberculosis had low blood levels of ascorbic acid, serum albumin, hemoglobin, vitamin A, carotene, and serum calcium in the order listed. All signs of nutritional deficiencies were more extensive in tuberculous than nontuberculous subjects. Persons with arrested tuberculosis had nutritive levels essentially the same as nontuberculous subjects. Farber & Miller have made a number of reports on their nutritional studies in tuberculosis. They report a prothrombin deficiency in 33 per cent of the nonbleeding and 53 per cent of bleeding tuberculous patients (142). Synthetic vitamin K restored the plasma prothrombin concentration but had no effect on the hemorrhage. In clinical studies, with regard to nicotinic acid and riboflavin deficiency, 25 per cent of 400 tubercular patients showed lesions suggesting deficiency (143). The principal types of lesions encountered were glossitis, cheilosis, and mucous membrane lesions, and they responded well to improved diet and specific vitamin therapy. Only seven of these 400 patients were found to have polyneuritis thought due to thiamine deficiency (144).

A nutritional finding of interest and possible value in the therapy of tuberculosis is that certain dietary changes will protect rats from the toxic effects of promin (sodium *p,p'*-diaminodiphenyl-sulfone-N,N'-didextrose sulfonate). The latter is an effective chemotherapeutic agent in experimental tuberculosis (145); however, it is quite toxic. The toxic effects include hyperirritability, anorexia, cyanosis, posterior paralysis, loss of hair, and a hemolytic type of anemia. Higgins (146) in self-selection experiments observed that young rats receiving promin developed a craving for some of the fractions of the vitamin B-complex—thiamine, riboflavin, and pyridoxine. Increased intake of these three vitamins was found to counteract the toxic effects of orally administered promin. In another report (147) it is stated that a diet



high in carbohydrate and containing whole liver was effective in protecting rats against promin toxicity.

Nutritional status and its relation to the pathogenesis of rheumatic fever has been the subject of several studies. It is known that the incidence of this common disease is higher in low income groups than among the well-to-do, higher in growing children than in adults, higher in urban than in rural communities. All of these factors influence environmental conditions including nutrition. Coburn & Moore (148) have compared the diets of fifty rheumatic children from families of low income with those of a like number of nonrheumatic children from families of high income. In such a comparison striking differences would be expected, and these were greatest with regard to protein, vitamin A, iron, and calcium. As "controls" in this type of experiment it would have been valuable to have nonrheumatic children from low income groups and rheumatic children from high income groups. In additional studies (149), they have re-investigated the relationship of vitamin A to the rheumatic state. While decreased plasma levels of vitamin A and carotene were found in those children with rheumatic fever, these were also the children with poor diets. The authors point out the well known fact that plasma vitamin A levels generally decrease in most acute infections and febrile conditions. As far as is known, vitamin A deficiency is not an important factor in the pathogenesis of rheumatic fever. Peete (150) has accumulated data on diet as a predisposing factor in rheumatic fever and concluded that the average diet of the patient with rheumatic fever and chronic rheumatic heart disease is low in foods which supply vitamins A and D and the minerals calcium, phosphorus, and iron. Although nutritional studies have not yet suggested that any specific nutrient is of value in the practical problems of the prevention or control of rheumatic fever, it is well established that most children who develop the disease generally have poor diets.

Interesting observations on diet and disease among the whites and the negroes of South Africa have been reported by Gilbert & Gillman (151). Cirrhosis and carcinoma of the liver, and tuberculosis are far more prevalent among the negroes, whereas gallstones, diabetes, and peptic ulcer are infrequent. Such difference in incidence of disease is undoubtedly related in part to racial characteristics and poor sanitation, but in addition, Gilbert & Gillman have considered nutritional status as a possible factor and present some experimental evidence to support this point. The negro is poor and lives largely on corn. Meat

and milk are rarely consumed; as a consequence, deficiency diseases are widespread. The effect of the typical negro diet on animals was tested over a two year study period using albino rats. As was to be expected, growth was poor in the test animals as compared to the controls receiving a mixed diet. After fifteen months, the test animals had lost much of their hair, their corneas were scarred and filled with capillaries, and numerous dental defects had occurred. On autopsy, liver lesions were present without exception. The testes of all male rats were extensively damaged. In many animals hypertrophy and hemorrhage of the adrenals were present. Atrophy of the thyroid was common while the parathyroids were usually enlarged. The parotid was always damaged while the pancreas was affected in a majority of the animals. Cardiac enlargement was present in those rats with extensive liver damage. In general, the observations were not those of any specific disease but rather of what might be expected as the result of chronic multiple deficiencies and as secondary manifestations of the extensive liver changes. This simple experiment demonstrates the possibility that long continued poor nutrition may have a close connection with many disease syndromes that are common today. It would indeed be unwise to dismiss nutrition as a factor in the cause or progress of most of the chronic diseases, especially as they occur in malnourished groups. Regardless of whether poor nutrition is a cause or effect in its relation to disease, the important fact remains that it frequently exists and hence should receive the best known treatments.

Perhaps the most striking illustrations of the improvement of health through better nutrition are to be found in studies on prenatal nutrition in relation to maternal health, fetal development, and the health of the offspring. This work in relation to man has been well substantiated by the recent papers of Burke *et al.* (152 to 155).

Smith (156) has recently commented on the place of nutrition in the field of public health and states that "no modern health unit can divorce itself from direct responsibility for the improvement of nutrition in the community which it serves." Unfortunately there has been and still is great reluctance on the part of public health leaders to assume this responsibility. Some of the reasons for this inertia and specific suggestions for the nutritional policy of health departments are given by Smith.

Parenteral administration of nutrients has a definite place in the practice of medicine and of surgery and is a phase of applied nutrition in which there is current interest and in which important developments

may be expected. Parenteral nutrition is desirable in conditions in which oral nutrition is impossible or is contraindicated, such as obstructive lesions of the gastrointestinal tract, ulcerative colitis, and in the early period following surgery of the gastrointestinal tract. Of the six groups of nutrients—water, minerals, carbohydrate, fat, protein, and vitamins—all have been given parenterally, at least on some occasions. The parenteral use of water, saline, and glucose has come into common use in the last two decades, and in the last year or so suitable preparations for the parenteral administration of some of the vitamins have become available. Recent developments in this field have centered on the parenteral administration of protein. Generally the protein source used has been a hydrolysate of casein, though more recently plasma protein, human albumin, and mixtures of synthetic amino acids have been employed. Elman has been one of the leaders in this field and many of his views together with references to his work are given in recent addresses (157, 158). Butler & Talbot (159) have reviewed the parenteral therapy of the patient unable to take fluid or food by mouth and present specific suggestions applicable to the parenteral therapy of infants, children, and adults. Attention is called to the inadequacies of present day parenteral nutrition therapy, and the oral administration of appropriate food early in the course of treatment is emphasized. Madden & Whipple and associates have infused a mixture of the ten essential amino acids plus glycine (160). They reported that this mixture is highly effective in plasma protein regeneration in dogs and is better tolerated than any protein digests they have tested. Further observations by these workers (161) suggested that the undesirable effects, such as nausea, vomiting, and pyrogenic reactions, occasionally caused by protein hydrolysates and certain amino acid mixtures, may be due to nonessential amino acids and indicated that glutamic acid is such an offender. Favorable clinical use of a mixture of pure amino acids has been reported (162).

The principal advantage that would be afforded by successful intravenous introduction of fat in addition to carbohydrate and protein would be in the high caloric value per unit volume and the avoidance of hypertonicity in the infusion solution. Many clinicians believe that the parenteral administration of fat is entirely unnecessary because for limited periods of time basal caloric requirements for patients at bed rest can usually be met by the use of 10 or 15 per cent glucose solutions. However, many patients in whom parenteral nutrition is desirable or necessary are in a poor state of undernutrition, frequently

cachetic. Nutritional requirements under such conditions, and also postsurgically, are not basal but increased. Thus a high caloric infusion mixture of reasonable volume and tonicity would be a valuable contribution to parenteral nutrition. Holt (163) has reported the intravenous administration of homogenized fat. Clark & Brunschwig (164) recorded the intravenous administration of glucose, casein digest, and emulsified fat throughout a seventeen day period in an adult male. Dunham & Brunschwig (165) have reported studies on the intravenous administration of homogenized olive oil and lard in dogs. Twenty-four animals were used in the study and infusions of fat were given equivalent to 2 to 3 gm. of fat per kilogram of body weight. Somewhat more than a third of the animals died as a result of the infusions. Large fat globules were found throughout many of the tissues and an anemia developed. McKibbin, Hegsted & Stare (166) have reported experiments on "complete parenteral nutrition" in which adult dogs were infused with mixtures containing all the nutritional factors known to be required by dogs. Corn oil emulsified with soybean lecithin was the fat used. Liver damage developed after twelve to eighteen daily infusions. It is possible that with improved methods for the preparation of fat emulsions better results may be obtained. Because the physiological arrangements for the absorption of fat differ from protein and carbohydrate in that the lymphatics play a special role, it is possible that factors in the intestinal wall necessary for fat metabolism are circumvented by the direct introduction of fat into the blood stream. Likewise, that fat so introduced would reach the systemic tissues prior to passing through the liver may be an important consideration.

#### NUTRITION SURVEYS

In discussing the series of surveys made on several portions of the Canadian population (55, 167 to 173), which have in general shown that 60 per cent of the population is "deficient," i.e., do not consume amounts equivalent to the RDA, McHenry (174) recognizes that the validity of these conclusions is affected primarily by (a) errors in calculations based on one week intakes, (b) errors in food composition tables, and (c) the fact that recommended allowances (used as requirements) may be too high. With regard to the latter fact, he mentions the following specific points: 45 or even 30 gm. of protein may be adequate; 12 mg. of iron is excessive for men; the riboflavin allowance is based on meager evidence; the thiamine allowance appears high and

evidence is not available to substantiate the levels allowed for various age groups; and 30 mg. of ascorbic acid may well be adequate. In addition, the diagnostic values of plasma levels of ascorbic acid are questioned as is corneal vascularization as an indication of riboflavin insufficiency. He believes that the chief value of the present surveys rests on the fact that they lead to a determination of the conditions which operate to produce poor nutrition and are an excellent means of health education. Re-evaluation of the results of one survey (172) using 0.23 mg. of thiamine per 1000 calories and 30 mg. of ascorbic acid as standards instead of the corresponding RDA, showed that by these standards 54 per cent of the girls received excellent diets compared to 16 per cent by the RDA standards, 35 per cent good diets compared to 55 per cent, 8 per cent fair compared to 22 per cent, and 1.7 per cent poor compared to 6.6 per cent. Such results appear more significant when compared to the physical examination scores, but since almost all the students were scored good or excellent on physical examination it is not possible to use the comparison as a test of the correctness of the dietary survey.

The Canadian surveys in general agree that the intakes of thiamine, ascorbic acid, and calcium are generally much below the RDA. Intakes of iron appear low in women and girls. A re-examination of one group (173) of students after two years failed to show any deleterious effects due to rationing and indicated some improvement, thought to be due to education and improved economic circumstances.

The exigencies of war have made England a peculiarly valuable proving ground in nutrition. A large population forced to live on diets restricted in certain nutrients is presented, together with a serious attempt by the proper authorities and scientists to determine the effects of this restriction. The diet is believed (56) to be primarily below current allowances in animal protein of which the ration allows about 20 gm. (meat protein) per day; in ascorbic acid, derived almost entirely from potatoes and cabbage, which amounts to about 20 mg. per day for the adult; vitamin A, of which carotene supplies the great majority and provides approximately 1500 I.U. per day; and in iron (discussed above). Aside from fairly clearcut evidence that the hemoglobin levels can be improved by iron administration, there appears to be no evidence of physical deterioration due to dietary deficiency. Therapeutic trials (57, 58, 60, 175) in fairly large groups have also failed to show a benefit from supplements other than iron. It is clear that health can be maintained for a period of years on far less

than the recommended amounts of the "seven basic foods," and convincing evidence for adequate levels below the RDA is being obtained.

The results of a nutrition survey in a rural county in North Carolina (2) shows that from 40 to 50 per cent of the people examined received less than one fourth of the RDA for ascorbic acid, over 50 per cent ingested less than one half the thiamine allowance, and 75 per cent received less than one half the riboflavin allowance. Calcium, iron, vitamin A, and caloric intakes were also far below the RDA. Clinical examination and several common laboratory methods, however, failed to indicate a comparable incidence of deficiency disease. The authors specifically do not evaluate the dietary intakes as to their sufficiency or insufficiency, but

one cannot avoid the suggestion that there exists a broad zone below the present National Research Council levels of recommended dietary allowances in which individuals can adjust themselves with no patent signs of deficiency disease. . . . When all this has been said, however, it is evident that improvement in diet is needed in this and similar communities and the pious hope is implicit that this would result in an increase in health, vigor, and vital living.

Youmans and associates (3, 176) reported that the caloric and protein intakes of a rural Tennessee population were much below the RDA although relatively little evidence for clinical deficiency could be found. In a more recent paper (177) the calcium intakes and the evidence for vitamin D deficiency are considered; of 1,124 subjects 171 were reported as consuming less than 0.3 gm. of calcium per day and from 68 to 87 per cent as less than the RDA. Only four women showed evidence of calcium deficiency. Roentgenographic examination revealed fifty-seven cases of mild osteoporosis but most of these cases were exhibited by women at or after menopause and may be unrelated to calcium intake. Studies on children also revealed few definite cases of rickets although the intakes of vitamin D were low. Only one sixth of the children from one to three years old received as much as 100 I.U.

The daily intake of various nutrients by women in low income groups in Austin, Texas, has been reported (178) as follows: calories, 1,145; protein, 33.8 gm.; calcium, 0.38 gm.; phosphorus, 0.72 gm.; riboflavin, 0.78 mg.; pantothenic acid, 2.4 mg.; nicotinic acid, 4.13 mg.; and thiamine, 0.49 mg. These authors report a fairly close correlation of physical findings with the analytic data. Thiamine deficiency was not observed. It was suggested that the lack of polyneuritis might be due to the low caloric intake, but it may be noted that the thiamine intake was above recent minimum values (66). Similar data on mod-

erate income groups have been reported (4) as follows: calories, 1,667; protein, 59.8 gm.; calcium, 0.93 gm.; phosphorus, 0.94 gm.; riboflavin, 1.71 mg.; pantothenic acid, 4.79 mg.; nicotinic acid, 9.07 mg.; and thiamine, 0.72 mg. Physical examination of these subjects was not made, but it was noted that caloric intake and weight showed no correlation.

The results of nutritional surveys in the United States have been summarized and discussed in a recent bulletin of the National Research Council prepared by a Committee of the Food and Nutrition Board (84). This report concludes:

All the evidence is in agreement that deficiency states are common among the population of the United States. Most of them are not the severe acute type. Rather they are less intense in degree and very much slower in their course. Predominantly the deficiency states here are mild, moderate, or severe chronic forms. Because of their slow gradual development, their presence is commonly unsuspected. In frequency and severity they increase with age and with lowered economic level. As yet optimum nutrition throughout the nation has not been achieved; on the contrary deficiency states are present on a large scale. . . ."

In this report the RDA have been interpreted as requirements, the hypotheses of Kruse (179) are accepted, plasma vitamin C levels below 0.6 mg. per 100 cc. are accepted as evidence of deficiency, and credence is given to corneal vascularization, xerosis conjunctivae, and gingivitis as specific signs of various deficiencies. The report has been criticized (180) and the statements with regard to the prevalence of deficiency states have been called "fantastic" (83). For the sake of the science of nutrition, and the respect with which it is considered by the medical and public health profession, it is unfortunate that this bulletin has been and is being very widely distributed, for many nutritionists and most clinicians interested in nutrition are not in agreement with the general tone and conclusions of the report.

The same committee discusses (181) some obvious and highly important principles underlying studies of nutrition pertaining to the influence of supplements on growth, physical fitness, and health. Ten conditions are given which should be considered in the planning and evaluation of nutritional studies. The committee is optimistic in stating that recognition and evaluation of these conditions will make "conflict and confusion give way to conformity and clarity." There are other conditions equally as important as those given and which are equally difficult to fulfill satisfactorily at the present time. To read the

report is a good lesson in self-discipline. The extensive bibliography is valuable.

Nutritional requirements, whether for man or animals, are difficult to discuss scientifically for such discussion involves consideration of what the requirements are for, i.e., growth, maintenance, health, resistance, etc., and these are terms which in themselves are difficult to define. Furthermore, it is known that requirements for any one nutrient depend to some extent on the amounts of other nutrients in the diet and on a host of other environmental factors. Relative requirements for most nutrients known to be required by man can be given but only with a considerable degree of variation and error. On the other hand, recommended dietary allowances can and should be made for most of the known nutrients. Such recommendations are useful as a goal for good nutrition and in the practical planning of dietaries provided they represent the best available evidence. The Food and Nutrition Board has done well in formulating the now widely publicized RDA. They state (1) that in using their recommendations, it is important that the purpose and general policies in formulating them should be understood.

The allowances for specific nutrients are intended to serve as a guide for planning adequate nutrition for the civilian population of the United States. The quantities given were planned to provide not merely the minima sufficient to protect against actual deficiency disease but a fair margin above this to insure good nutrition and protection of all body tissues. Since the actual requirements for these purposes are not known, it is recognized that the margins of safety may vary considerably for the different factors.

However, the Board has not always followed its own advice for its widely circulated Bulletin 109 states "all the data from numerous surveys . . . are entirely in accord in showing that deficiency states are rife throughout the nation." And most of the data from most of the surveys imply deficiency states principally on the basis of not meeting the RDA. In general, the misuse of the table has been along those lines, i.e., on the false assumption that not meeting the RDA is evidence of inadequate dietary intake and of malnutrition. The Board could aid the situation greatly by making a stronger attempt to correct the misuse of the table.

Clinical examinations of most of the subjects in the various surveys fail to show more than a few cases of ill health. Most of the survey reports mention this lack of correlation between dietary findings and clinical and laboratory data. In discussing this situation, Dann (182)



has pointed out that "it is logically indefensible to speak of failure 'to obtain consistent correlation between dietary studies and findings on medical examination,' since the dietary intake and the clinical condition are related as cause and effect." He states further that if opposing conclusions are drawn from the two sets of data, it can only mean "that the underlying logical process is faulty," and that "ultimately, all statements concerning the presence or absence of vitamin deficiencies must rest on clinical (including biochemical and physiological) findings, and not on food intakes unless they can be equated with clinical findings."

Sinclair's (183) interesting discussion also stresses that "we must place our emphasis on health in man, rather than on isolated abstractions culled from work on lower animals." While this statement is undoubtedly true in the final appraisal of the nutritional status of man, one should give full credit to the brilliant work in animal nutrition which really "made" the science of nutrition, and without which one would have little understanding of human nutrition.

These quotations, to which many more might be added, foreshadow the trend in human nutrition today. In nutrition, as in all forms of human endeavor recorded in history, the "gospel" must and will eventually be displaced by the scientific evidence. Perhaps the greatest needs in nutritional research are adequate and objective methods for the evaluation of health.

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## WATER-SOLUBLE VITAMINS

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During the past year water-soluble vitamins have again proved to be a fertile field of investigation. The reviewers, as a consequence, have had to eliminate from consideration many papers of interest to research workers in this field either because they were not entirely pertinent, contained results which were largely negative or were of too preliminary a character. Some papers also may have been omitted unavoidably because the journals were not available to the reviewers. An attempt has been made, however, to make the review as inclusive as possible in view of the present breadth of interest in water-soluble vitamins.

### ASCORBIC ACID

*Chemistry.*—A crystalline acetyl derivative of ascorbic acid has been prepared by the action of ketene and isopropylidene ascorbic acid in anhydrous acetone. Its structure was shown to be 3-acetyl-5, 6-isopropylidene ascorbic acid (1).

A canary yellow color has been produced when a mixture of ascorbic acid and nicotinamide or nicotinic acid was moistened with water and stirred into a thick paste (2). In further study morpholine, piperazine, and various pyridine and quinoline derivatives were also found to give the yellow color. The reducing properties of ascorbic acid were found to be unchanged by the color reaction with nicotinamide (3).

In a study on the use of *d*-isoascorbic acid and its sodium salt as antioxidants, *d*-isoascorbic acid was oxidized more rapidly than the sodium salt and, in contrast to the latter, protected *l*-ascorbic acid from oxidation (4).

Further evidence has been obtained of the positive effect of ascorbic acid and of iron upon the oxygen uptake of tissue suspensions and phospholipids. Ascorbic acid and very small amounts of iron together caused a very great acceleration of the oxygen uptake of brain and

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liver suspensions and of phospholipids. No other reducing agent was able to replace ascorbic acid in the system (5). In an *in vitro* study of the reduction of methemoglobin by ascorbic acid, methemoglobin produced by ferricyanide was reduced more rapidly by ascorbic acid than that produced by nitrite. This difference appears to be due to the catalytic effect of ferricyanide on the reduction of methemoglobin rather than to a chemical difference between the two preparations (6).

The results of experimental work on the mechanism for the development of oxidized flavor in milk have indicated that ascorbic acid forms either a complex or a direct compound with copper ion. Citric acid was observed to inhibit the destruction of ascorbic acid by copper ion. This appeared to be caused by the development of a copper complex with citric acid which removed the cupric ion from the reaction (7).

The synergism of ascorbic acid with quinone in the stabilization of ethyl esters of lard fatty acids has been shown to be due to the fact that the quinone catalyzes the oxidation of the vitamin. The reactions initiated by this result in the reduction of the activated fat peroxide radicals and thus the substrate is protected (8).

Thirty species of plants have been examined for the presence of an enzyme catalyzing the reduction of dehydroascorbic acid by reduced glutathione. The addition of the juice from nine species doubled the rate of reduction of dehydroascorbic acid caused by glutathione alone. The most active species were cauliflower and broad bean, in both of which glutathione protects ascorbic acid from oxidation by atmospheric oxygen (9).

Both the oxidized and the reduced forms of ascorbic acid have been found to interfere with the determination of sulfanilamide by the method of Bratton & Marshall, when the ratio of ascorbic acid to sulfanilamide is one to two or greater. Complete inhibition of the reaction did not occur, however, until the ratio reached the value of twenty-five to one or greater (10).

*Assay.*—Two methods for the rapid determination of ascorbic acid in plant materials have been reported. One represents an adaptation of Stotz's method for the estimation of ascorbic acid in blood and urine, dependent upon the quantitative extraction of the unreduced 2,6-dichlorophenol indophenol dye from an acid aqueous solution by xylene. The method appeared to be applicable to all plant materials assayed, whether fresh, frozen, or dehydrated, and to be especially

useful for highly colored or turbid extracts (11). The other method involves titration with a rather concentrated solution of 2,6-dichlorophenol indophenol using a microburet. Evidence was obtained that 1 to 3 per cent metaphosphoric acid can be used for titration with no loss of ascorbic acid due to insufficient extraction and without the danger of destruction of the dye by the extracting acid (12).

The original 2,4-dinitrophenylhydrazine method for the determination of the total ascorbic acid in blood and urine has been adapted to the direct determination of dehydroascorbic acid in the presence of ascorbic acid in plant tissue. The dehydroascorbic acid of a metaphosphoric acid extract, in which the ascorbic acid is stabilized with thio-urea, is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative treated with sulphuric acid to produce a red color which is measured photometrically. Certain modifications were also made in the dinitrophenylhydrazine method in order to make it suitable for the determination of ascorbic acid in plant tissue (13).

Lugg's procedure designed to improve the specificity of the indophenol titration has been critically examined. Improved specificity was obtained provided certain conditions were controlled. Glycine, reductone, and reductic acid and certain unknown materials in various extracts were found to introduce some error, but the error was reduced by decreasing the reaction time employed (14).

Ascorbic acid has been found to be inert to indophenol or iodine in the presence of strong hydrochloric acid, but glucoreductone and other interfering substances were reduced under these conditions. On dilution with water, the usual reaction occurred between indophenol and ascorbic acid. This made possible the determination of ascorbic acid in the presence of glucoreductone by differential titration (15).

A method for the elimination by means of hydrogen peroxide of the interference of sulphur dioxide in ascorbic acid titrations has been developed. Under the conditions used, hydrogen peroxide had no appreciable effect on ascorbic acid unless iron or copper salts were present (16). The dye titration method with indophenol used for estimating blood ascorbic acid has been found to yield values which are too high due to the inclusion of a variable amount of extraneous reducing substance. A simple lead precipitation eliminated this error (17).

A new bioassay for ascorbic acid has been reported which is based upon the increase in serum alkaline phosphatase of scorbutic guinea pigs following administration of a critical dose of ascorbic acid. Sub-

stances of known and questionable potency as determined by other methods were assayed successfully by the new bioassay (18). Another procedure for the bioassay of ascorbic acid has been developed which involves the measurement of the size of the odontoblasts of the decalcified teeth of guinea pigs fed daily doses of the vitamin varying from 0.5 mg. to 2 mg. (19).

*Tests for nutritional status.*—A further study has been made of the saturation test as a means of determining whether or not a dietary deficiency of ascorbic acid exists. Satisfactory results were obtained with a 200 mg. dose of ascorbic acid. A urinary excretion of approximately 20 mg. or more of ascorbic acid in four hours or approximately 30 mg. in six hours was believed to be indicative of an adequate ascorbic acid diet. Less than 10 mg. in six hours was definitely considered inadequate (20). A simplified modification of the method of employing responses to a test dose has been used for the determination of saturation needs of ascorbic acid. Only the twenty-four hour samples of urine before and after the test dose were analyzed. Of nine young women, six were found to have an ascorbic acid requirement for saturation between 1.4 mg. and 1.8 mg. per kg. (21).

The plasma ascorbic acid concentration was reported to be a satisfactory index of the nutritional status with respect to ascorbic acid, unless the subject had been on a higher intake of ascorbic acid previous to the test (22). A test for ascorbic acid status based on the intradermal injection of a dilute solution of dichlorophenol indophenol which is decolorized by the vitamin has been proposed. The skin test paralleled the degree of body saturation (23).

*Surveys of nutritional status.*—Investigations have been made of the ascorbic acid status of various groups of individuals. In a Puerto Rican study, 57.9 per cent of the subjects had plasma ascorbic acid values in the severe deficiency range (24). In a study in Newfoundland, approximately 72 per cent of the subjects were found to be in a deficiency state. The ascorbic acid blood plasma levels showed a marked seasonal variation which paralleled the available ascorbic acid sources in the diet (25). The results of a study on pregnant women in war time showed that the saturation, although varying widely, was on the low side. The patients became definitely more saturated during the summer months (26). A study on university women showed that almost 25 per cent of the freshmen and 10 per cent of the upper classmen had low ascorbic acid blood plasma levels (27). In a study of hospital patients evidence was obtained of suboptimal intake of ascor-

bic acid which was most serious during the early months of the year (28).

Analytical and statistical data obtained from surveys conducted during the winter of 1941-42 have shown that about 88 per cent of the men in one unit of the Canadian Regular Army and 70 per cent in another had an estimated daily ascorbic acid intake of less than 30 mg. from vegetable sources. Current methods of cooking were found to leave only a small fraction of the original ascorbic acid in the food served (29).

*Experiments on human requirements.*—In a study of daily ascorbic acid requirements of the normal human adult, weekly averages of daily plasma ascorbic acid values have been found to give a better indication of the vitamin status of the individual than a single determination. Utilization values in urinary excretion studies were shown to be close to 1 mg. per kg. of body weight. An intake of 1 mg. per kg. of body weight was shown to increase plasma ascorbic acid values for all subjects studied and several were shown to reach saturation on such a retention (30).

The probable minimal daily amount of ascorbic acid needed by healthy male adults has been found to lie in the neighborhood of 75 mg. as gauged by the response of the plasma ascorbic acid values and health of the gum tissues. Refractory individuals with low grade chronic infections such as nose and throat infections or deeply diseased gums appear to need at least 100 mg. daily to obtain a satisfactory response (31).

A daily intake of 23 to 25 mg. of ascorbic acid has been shown insufficient to maintain tissue saturation in pre-school children. Evidence was obtained that the ingestion of potassium citrate increased the utilization of ascorbic acid in some of the children. On substituting orange juice for crystalline ascorbic acid and potassium citrate, the utilization of ascorbic acid was still further increased (32).

*Other experiments with humans.*—Reduced and total ascorbic acid urinary excretions have been determined following intravenous injection of ascorbic acid. The excretion of total ascorbic acid paralleled the excretion of reduced ascorbic acid. The difference between the total ascorbic acid and the reduced ascorbic acid excretion was highly significant (33).

Approximately 60 per cent of the patients suffering from chronic ulcerated colitis have been found to have low fasting ascorbic acid plasma levels and about 66 per cent of the patients studied for capillary

fragility showed abnormality (34). Although this appears to be evidence of correlation between low fasting ascorbic acid plasma levels and capillary fragility, a statistical study of 214 simultaneous determinations of capillary resistance and plasma ascorbic acid provided no support to the view that capillary resistance is positively correlated with ascorbic acid levels (35).

Ascorbic acid given orally has been observed to result in a small diuresis in patients with cardiac decompensation. When injected intravenously, however, no appreciable similar effect was observed. In combination with mercupurin the diuresis was considerably greater than with mercupurin alone (36).

In seven cases of total disruption of surgical wounds of the abdominal wall, ascorbic acid could not be detected in the blood plasma at the time of total disruption. In five cases the administration of 150 mg. of ascorbic acid a day was inadequate to produce detectable quantity in the plasma within one week. One patient received 150 mg. per day for six weeks before the presence of ascorbic acid in the plasma was observed. Another patient had no detectable plasma ascorbic acid after four weeks administration (37).

*Guinea pig experiments.*—A single random determination of the reduced ascorbic content of the plasma of guinea pigs has been reported not to be indicative of the state of ascorbic acid nutrition of the animal. Individual variation and the time elapsing between the last intake of the vitamin and the withdrawal of the blood sample from the animal were found important (38).

In a study of the relation of the blood concentration of ascorbic acid to the tissue concentrations and to the histology of the teeth of the guinea pig the most efficient rate of withdrawal of ascorbic acid from the blood by various tissues occurred at a whole blood concentration of almost 0.25 mg. per 100 ml., but a concentration of ascorbic acid in the tissues reflected by a blood level above 0.22 mg. per 100 ml. prevented the appearance of any pathological lesions in the incisor teeth. An intake of 1.23 mg. of ascorbic acid per 100 gm. of body weight was required in the guinea pig to produce a whole blood level of 0.25 mg. per 100 ml. (39).

Observations have been reported that a scorbutic diet fed to guinea pigs retarded the deposition of bone salt and caused a reduction in the phosphatase present at the costochondral junctions. The administration of pure ascorbic acid permitted normal calcification (40).

In a comparative study of neuromuscular regeneration in guinea

pigs subsisting on different levels of ascorbic acid, the regenerating muscles of animals given suboptimal amounts of the vitamin were found to be relatively weaker than those of animals supplied with adequate amounts. Excess intakes of ascorbic acid had no beneficial effect upon the course of neuromuscular regeneration (41).

The effect of ascorbic acid deficiency on fat metabolism in guinea pigs has been studied using the paired feeding technique. No significant differences were noted in the gross amounts of liver, adrenal, or carcass lipids in the scorbutic and control guinea pigs, but the livers and adrenals of the scorbutic animals were significantly larger (42).

Results have been obtained which demonstrate the *in vitro* oxidation of *l*-tyrosine by liver slices from normal guinea pigs and the inability of the same tissue from ascorbic acid-deficient animals to exhibit similar metabolic activity. Kidney tissues showed only a little tendency to oxidize the amino acid, although also reflecting the state of ascorbic acid nutrition of the test animal (43).

Contrary to previous findings the fibrinogen content of guinea pig plasma has been observed to increase markedly with the onset of scurvy. The restoration of normal fibrinogen levels in scorbutic guinea pigs followed within two weeks after the basal diet was supplemented with raw cabbage or *l*-ascorbic acid (44).

The extent and duration of the hypoprothrombinemia induced by 3,3'-methylenebis (4-hydroxycoumarin) have been reported to be markedly increased in scurvy in guinea pigs. Single 100 mg. doses of *l*-ascorbic acid did not protect nonscorbutic animals from the hypoprothrombinemia but repeated 100 mg. doses of the vitamin exerted a detectable protective effect (45).

Contrary to the results of previous work on humans, results have been obtained during the year which indicate that ascorbic acid does not reduce the toxicity of sulfanilamide for guinea pigs (46). A relationship between total blood ascorbic acid and tensile strength of wounds in guinea pigs has been established (47). The epinephrine content of the adrenal glands of guinea pigs in scurvy, on the other hand, is significantly increased (48). Evidence has been presented that the insulin content of the pancreas of guinea pigs decreases markedly in scurvy (49).

*Experiments with other animals.*—The ascorbic acid content of the plasma of the mare has been determined over a period which included a change from winter to summer pasture rations. The mean value for the entire period was  $0.53 \pm 0.17$  mg. per cent and the values for

the prepasture and pasture periods were  $0.42 \pm 0.17$  and  $0.59 \pm 0.16$  mg. per cent respectively (50).

Variation in rations has been shown to affect the plasma ascorbic acid values of feeder lambs. Alfalfa hay and yeast were found to cause the most marked increase in plasma ascorbic acid values, while a ration composed of oats, straw, silage, and corn had the least influence. The mean plasma ascorbic acid value for suckling lambs was found to be  $0.48 \pm 0.21$  mg. per 100 ml. (51).

Ascorbic acid has been reported to improve the growth of chicks when added to a purified diet. The feeding of arabinose or *p*-aminobenzoic acid gave responses similar to that of ascorbic acid. Ascorbic acid counteracted somewhat the depressing effect of succinylsulfathiazole when fed with the basal diet, but was not as effective as *p*-aminobenzoic acid in this respect (52).

In a study in which seven strains of mice were examined the ascorbic acid content of the livers of pregnant mice was found to be higher than in nonpregnant animals (53).

A high concentration of ascorbic acid has been demonstrated in the walls of the intestines of specimens of *Belascaris marginata* taken from dogs fed a normal diet. The parasite appeared to be able to absorb considerable quantities of ascorbic acid from the food of the host (54).

*Hormones and ascorbic acid.*—Pure adrenotropic hormone has been found to diminish the ascorbic acid content of the adrenal of the rat within a short time after the administration of a single dose (55). The feeding of iodinated casein to milking Holstein cows in the latter part of their lactation caused the ascorbic acid content of the milk to decrease to two thirds of normal (56). Contrary to some of the results previously reported, the simultaneous injection of ascorbic acid and pregnant mare serum gonadotropin into both young and old rats failed to cause a greater increase in weight of the testes, ovaries, or uterus than did the injection of gonadotropin alone (57).

The administration of various gonadotropic hormone preparations has been found to reduce the blood plasma ascorbic acid in ewes and the administration of a preparation from pregnant mare serum has been observed to have a similar effect in rabbits (58).

*Disease and ascorbic acid.*—Using hemolysis as the index of complement activity, no significant change has been observed as a result of partial or complete deficiency of ascorbic acid (59). The pathological processes, involved in the healing of staphylococcal abscesses in

scorbutic guinea pigs, have been found to be quite different from those of normal, paired control animals (60). Ascorbic acid has been reported to cause a tremendous inactivation of purified influenza virus by means of tests with chick embryos and mice (61).

In a comparative study of ascorbic acid content of tumors and homologous normal tissues no correlation has been observed between the ascorbic acid content of tumors and that of the tissue of origin, rate of growth of tumor, age of host, or site of transplantation (62). The mean concentration of ascorbic acid in the liver of mice of three high-cancer pure lines was found to be greater than in the low-cancer pure lines and in other low-cancer breeds (63). The injection of certain carcinogenic compounds intraperitoneally has been observed to cause an increase in the concentration of ascorbic acid in the liver of mice (64).

*Miscellaneous.*—Dehydroascorbic acid has been found to be present in many instances in thermal sweat, but the amounts were not sufficient to suggest the production of ascorbic acid deficiency through excessive sweating (65). The results of an investigation of the relationship of the ascorbic acid content of grapefruit to the nitrogen nutrition of the tree have shown that the fruit from trees handled to give a low nitrogen content at harvest are higher in ascorbic acid content than those from trees in which a higher nitrogen plane prevailed (66). Ascorbic acid has been observed to affect the volume and improve the grain texture and crumb color of loaves of bread in a manner similar to potassium bromate (67). *Phaseolus radiatus* seeds germinated in distilled water to which certain minimal concentrations of manganese were added have been observed to show greatly increased synthesis of ascorbic acid (68).

#### THIAMINE

*Biochemistry.*—Acetylation of sulfanilamide was found to be defective in rats with thiamine deficiency. The defective acetylation was undoubtedly due to retarded oxidation of pyruvic acid which automatically decreased the formation of acetyl phosphate with consequent inhibition of acetylation (69).

*Assay.*—Assay methods for the determination of thiamine continued to hold the attention of workers in this field. The reports during the past year deal chiefly with improvements and modifications of the various methods. Evidence was obtained that the natural thia-



mine of wheat does not occur as cocarboxylase and that an enzyme digestion in the thiochrome assay procedure for wheat flour is not required for the estimation of the natural vitamin. The presence of a heat-destructible factor capable of hydrolyzing cocarboxylase was demonstrated in flour (70). The rates of destruction of thiamine in phosphate and phthalate buffers increased markedly as pH increased, but at a given pH the phosphate buffer allowed less decomposition than did the phthalate buffer. The rate of destruction of cocarboxylase was faster than the rate of destruction of thiamine at a given pH. The rate of destruction was also influenced by traces of various metals (71). A method was described for the simultaneous determination of thiamine and nicotinamide methochloride (72).

*Thiochrome method.*—In the determination of thiamine by the thiochrome method suggestions have been made for overcoming the effects of temperature and dissolved oxygen on the fluorescence of the quinine standard and of thiochrome (73). Uptake of thiamine by yeast and subsequent extraction was reported as eliminating difficulties involved with the thiochrome method due to interfering blue fluorescent compounds (74). Certain electrolytes were found to have a quenching effect on the fluorescent intensity of thiochrome (75). An improved thiochrome method for the determination of thiamine in urine was reported which permits the accurate determination of thiamine in the presence of the fluorescent pigment  $F_2$ , a derivative of nicotinic acid which at times causes major errors in the determination of thiamine (76). The accuracy of simplified procedures for the determination of thiamine in wheat flours, bread, and other cereal products compared favorably with that of the regular method (77, 78).

A micro-method has been developed for the estimation of thiamine by making a special modification of the thiochrome method. In solutions of pure thiamine or in cereal extracts the method was accurate to  $\pm 20$  per cent at concentrations in the region of 0.001  $\mu\text{g.}$  the accuracy increasing to  $\pm 3$  per cent at 0.05  $\mu\text{g.}$  (79).

A method for the determination of thiamine in urine after its conversion into thiochrome by means of sodium hydroxide and potassium ferricyanide has been described (80). A simple adaptation of the Melnick-Field colorimetric method for the determination of thiamine in urine has made possible the use of the 4-hour thiamine clearance test, which is regarded as the most convenient and versatile in diagnosing thiamine deficiency (81).

*Microbiological methods.*—A microbiological assay procedure has

been developed for the determination of thiamine based on the growth response of *Lactobacillus fermentum* 36 to this vitamin. Heavy growth was secured with thiamine but there was no response to similar amounts of the pyrimidine and thiazole halves of the thiamine molecule either alone or together or in the presence of thiamine or biological extracts (82). Pyrithiamine, the pyridine analogue of thiamine, and 6-aminopyrimidine compounds inhibited the utilization of diphosphothiamine for growth of *Lactobacillus fermentum* and *Penicillium digitatum* more than they inhibited the use of thiamine. The results indicated that cocarboxylase formed from thiamine is a more firmly bound enzyme than that formed from added diphosphothiamine and suggested that thiamine is attached to the apoenzyme before being phosphorylated (83).

Thiamine and pyrimidine have been reported to stimulate carbon dioxide production by yeast. This capacity varied with the concentration of these compounds, thiamine being more stimulatory than pyrimidine at high concentrations, with the reverse obtaining at the lower concentrations. Cocarboxylase, thiazole, and pyrimidinesulfonic acid as well as oxychlorothiamine and certain phosphate esters stimulated yeast fermentation (84). Suggestions have been made for the elimination of sources of error connected with the use of the macro fermentation method for the assay of thiamine in meat and egg products (85). The yeast-stimulating activity of plasma as determined by the yeast-fermentation method was found to vary with the level of thiamine excretion and with the true plasma thiamine concentration. Thus measurements of the yeast-stimulating activity of plasma were suitable indices of thiamine nutrition (86). Interference of heavy metal salts and certain other compounds in the determination of thiamine with the diazotized *p*-aminoacetophenone reagent resulted in lower values in the colorimetric determination of the vitamin. The extent of the phenomenon is a function of the concentration of the extraneous compound (87).

*Comparison of assay methods.*—In a comparative study of yeast-growth, yeast-fermentation, and thiochrome methods the latter appeared to be a satisfactory means of determining the thiamine content of most natural and processed materials. In the case of processed materials the yeast-growth method gave very high results. With certain limitations the yeast-fermentation values agreed with the thiochrome results (88). Upon assaying twenty-nine pharmaceutical products no marked differences were observed in the values obtained by

the rat-curative, thiochrome, and fermentation methods. The thiochrome and fermentation methods agreed better than any other two methods although the two biological procedures seemed to give higher values than the thiochrome method (89). The values for thiamine in beef muscle obtained by the fermentation procedure checked those obtained by the thiochrome reaction with application of the adsorption procedure. The values were considerably lower than those obtained without adsorption (90). The results of a collaborative study on the thiamine assay of enriched flour gave an average value of 2.11 mg. per lb. which is in close agreement with that calculated from an assay of unenriched flour and the added amount of pure thiamine, namely 2.09 mg. per lb. (91). In an application of the Prebluda-McCollum reaction in the chemical determination of thiamine and cocarboxylase in animal tissues, grain cereals, and human feces, the thiamine values obtained were between 10 and 15 per cent lower than those obtained by the thiochrome method (92).

*Interrelationships.*—A number of interrelationships have been shown to exist between thiamine and other vitamins and nutritive factors. Chronic thiamine deficiency, in agreement with acute thiamine deficiency, produced great losses of riboflavin in urine, uncomplicated by body tissue catabolism, and hence lowered the retention of the latter vitamin (93). An interrelationship between thiamine and riboflavin has been revealed by the fact that in rats depletion in thiamine was found to increase the concentration of riboflavin in the liver and kidneys above that of control animals (94, 95). Both thiamine chloride and thiamine pyrophosphate in low concentrations slightly increased the synthesis of acetylcholine (96). Thiamine chloride and related compounds had no direct effect on the striated muscle of frogs in low concentrations. In higher concentrations the effect of acetylcholine in inducing muscle contraction was depressed. The thiamine compounds significantly increased the effect of potassium in inducing muscle contraction (97).

*Carbohydrate metabolism.*—In a study with dogs dealing with carbohydrate metabolism in thiamine deficiency the postabsorptive level of blood sugar became high in most instances. Both lactic acid and pyruvic acid accumulated in the blood of thiamine-deficient animals (98). The rate of intestinal absorption of galactose in the rat was not affected by suboptimal intakes of thiamine (99). In the synthesis of fatty acids from carbohydrate precursors it was found that the decrease in fat content in rats on thiamine-deficient diets resulted from

failure of synthesis and deposition of fatty acids, and that this failure was attributable chiefly to the diminished food intake rather than to any specific action of thiamine. The fact that saturated fatty acids were found to be consistently richer in deuterium than the singly unsaturated fatty acids was taken to support the belief that the saturated acids are the primary products of fatty acid synthesis in rats, and that oleic and palmitoleic acids are formed from these by secondary dehydrogenation (100). The fact that administration of thiamine to rats fed casein alone and glucose alone prolonged their lives 66.7 per cent and 97.3 per cent, respectively, above life length without the thiamine supplement, was interpreted as showing that thiamine plays an important role in the utilization of protein as well as carbohydrates (101).

*Endurance.*—Muscular endurance which was greatly decreased on a diet, which was low in the B-complex vitamins, was increased significantly when B-complex vitamins in the form of a natural source such as whole wheat bread or pure thiamine hydrochloride were added to the diet. The effect of the bread seemed to be due chiefly to the thiamine (102). Thiamine deficiency, as shown by a unique method of measuring quantitatively the work performance of rats by their swimming ability, resulted in a rapid and marked decrease of physical fitness (103).

*Effects of deficiency.*—In thiamine deficiency the cat has been observed to develop symptoms like those in most animals. Of special interest, however, was the observed impairment of the labyrinthine righting reactions. The inability to right in midair and other symptoms led to the conclusion that the functional lesion of thiamine deficiency was in the synaptic centers of the midbrain (104). Resistance to low oxygen tension was also increased in thiamine deficiency in cats (105).

Cats fed an exclusive diet of raw carp or raw herring developed all the signs of thiamine deficiency characteristic of this species. Administration of pure thiamine alleviated the condition (106). The destruction of thiamine by the fish principle in fish tissue has been shown to be an enzymic reaction (107). The destruction was markedly influenced by certain metallic ions and by compounds resembling the thiamine molecule (108, 109). In the enzymatic reaction a hydrogen ion was produced which may arise from the water molecule involved in the formation of an alcohol. It was suggested also that the fish principle is unique among those enzymes catalyzing hydrolytic reactions (110).

The relation of thiamine to blood regeneration was shown with dogs maintained on a highly purified ration deficient in thiamine. Inanition, resulting from thiamine deficiency, when associated with the strain of phlebotomy, caused some limiting of the hematopoietic ability of the animal, which was not present on a restricted thiamine intake (111). The concentration of total and phosphorylated thiamine in the liver of dogs was observed to rise during prolonged hemorrhagic shock. The increase in liver thiamine seemed to be related to the duration of shock. The nonphosphorylated thiamine of muscle also rose markedly during hemorrhagic shock. This change occurred at the expense of the cocarboxylase (112).

Pigs in which chronic thiamine deficiency was produced showed loss of appetite, vomiting, and impairment of growth but the results failed to support the claim that lack of thiamine causes degenerative changes in the nervous system (113). With natural diets of different thiamine content and synthetic rations with different amounts of pure thiamine added, it was found that the amount of thiamine deposited in the body was directly related to the thiamine intake. Results were also obtained which suggested differences in intestinal vitamin synthesis between pigs fed a synthetic as compared to a natural ration (114).

Monkeys with thiamine deficiency showed a drop in weight, decreased food consumption, general muscular weakness, loss of reflexes, convulsions, incoordination, increasing cachexia, signs of cardiac insufficiency, prostration, and death (115).

Deficiency of thiamine in the diet increased the resistance of mice to the Lansing strain of poliomyelitis. Both mortality rate and the incidence of paralysis were lower in the deficient animals than in the normally fed controls. The effect of a deficiency appeared to be more in delaying the action of the virus than in preventing it (116, 117, 118).

*Clinical observations.*—Human intoxication has been reported in infants fed milk of thiamine-avitaminotic women. The intoxication was characterized by vomiting, abdominal pains, diarrhea, stiffness of neck, and convulsions. In the most acute form the attack consisted of dyspnea, cyanosis, and running pulse. The action apparently was due to intermediary metabolites which appear to be synergic and cumulative (119). Sensitization to thiamine hydrochloride has been reported in man. The response of a patient to parenteral administration of thiamine followed the familiar pattern of sensitization to proteins

(120). A case of congenital and infantile beriberi has been observed in which the mother, despite an inadequate diet, did not show any marked clinical findings of beriberi, but in which the infant was born with severe, almost fatal manifestations which responded spectacularly to thiamine administration (121).

In subclinical thiamine deficiency induced in normal young men clinical symptoms were found to be minimal and not in themselves sufficient for diagnosis. However, measurable changes were observed in the yeast-stimulating activity of samples of fasting plasma and of skeletal muscle, in the urinary excretion of thiamine, and in the metabolism of pyruvate. A measurable decrease in both extracellular and intracellular thiamine concentrations was detected (122).

*Requirements.*—The thiamine requirement of children of pre-school age was reported as being approximately 0.50 mg. per 1,000 calories. It was suggested that the return of 12 per cent of a test dose of thiamine or fasting 1-hour excretions of 6  $\mu$ g. be considered indicative of satisfactory nutritional status (123). The thiamine requirements of young adult males were shown to be far lower than supposed. Since biosynthesis may act as a protective mechanism, and may vary in its action with diet, the evaluation of the thiamine requirements appeared to be necessary on each particular diet (124). The amount of carbohydrate in the diet was found to be an important factor in determining the daily human requirement for thiamine. Urinary excretion of thiamine was decreased when the carbohydrate-fat ratio in the diet was increased. Decreased thiamine excretion indicated increased need in metabolism. No evidence of a thiamine-sparing action of fat was observed (125).

The minimum thiamine requirement for maintenance of monkeys weighing about three kilograms was determined to be 15  $\mu$ g. per kg. or 40  $\mu$ g. per day. The minimum requirement for growth was 25 to 30  $\mu$ g. per kg. per day or 75 to 100  $\mu$ g. per day (115).

The thiamine requirement of young pigs has been shown to be related to the fat content of the diet. As indicated by failure in appetite and cessation of growth, the animals on the low level of fat showed evidence of thiamine depletion on the average in twenty-five days, those on the medium level in twenty-eight days, and on the high level in thirty-three days (126).

An increased thiamine requirement for rats in hot environments has been reported. Diets containing 1 mg. of thiamine per kg. of food gave optimal growth at 68° F., while for the same growth rate at 90

to 91° F. 2 mg. of thiamine were needed. The requirement was not increased until the rats had been kept for at least two weeks in the hot environment (127).

*Miscellaneous.*—On increasing doses of thiamine it was found that at the higher levels of intake the efficiency of utilization decreased. Part of the losses was caused by destruction in the tissues (128).

No harmful action was noted in the effects of massive doses of thiamine on fertility and lactation in the albino mouse for three generations, even though at times the consumption of thiamine per mouse equalled 2 mg. per day (129).

The rate of thiamine destruction appeared to be influenced by amino acids and related compounds (130). The difference in thiamine utilization of rats maintained on diets containing dextri-maltose or sucrose suggested the presence of factors in the autoclaved yeast and dextri-maltose which allowed greater utilization of or supplemented the thiamine present (131).

Thiamine has been shown to be connected with the metabolism of estradiol by liver slices since those from thiamine-deficient rats were unable to inactivate estradiol under conditions in which slices from controls on the same diets, but receiving adequate amounts of the vitamin, possessed this activity. This loss of inactivating ability paralleled the change of thiamine content in the liver (132, 133). The rate of alcohol metabolism was shown not to be decreased during thiamine deficiency (134).

The total amount of thiamine in fertile hen eggs assayed by the macro fermentation method did not change during incubation. It was also found that the thiamine content did not change in sterile eggs kept at incubation temperatures for three weeks or in eggs kept for this period at room temperatures and at 5° C. (135).

#### RIBOFLAVIN

*Biochemistry.*—A deficiency state, produced in bacteria and mice with phenazine analogues of riboflavin, was overcome by sufficient amounts of riboflavin (136). Acetylation of sulfanilamide was incomplete in rats with riboflavin deficiency. This finding indicated that the lactic acid dehydrogenase system was defective with the result that lactic acid is not converted to pyruvic acid, the decreased formation of which would account for the imperfect acetylation (69). The feeding of isoriboflavin to rats receiving a sub-optimal intake of riboflavin

inhibited the growth-promoting action of the vitamin. The antagonistic effect of the isomer was prevented or overcome by the feeding of an adequate level of riboflavin (137).

*Assay.*—A number of factors have been shown to have a bearing upon the refinement and standardization of riboflavin determinations. Riboflavin solutions were affected by various lighting conditions such as exposure to direct sunlight, diffused daylight, artificial light, and ultraviolet light. A 150 watt reflector screened with a red cellophane filter did not affect adversely aqueous acid riboflavin solutions and provided adequate illumination in the laboratory (138). Certain electrolytes were found to have a quenching effect on the fluorescence intensity of riboflavin (75).

Other factors affecting the determination of riboflavin by the fluorometric method were treatment with Florisil and permanganate (139, 140). A simplified fluorometric method for determining riboflavin has been reported (141) and a rapid method for the determination of riboflavin in wheat and wheat products (142). A modification of the microbiological method of Snell & Strong has been developed which can be used for the determination of amounts of riboflavin in the range of 0.5 to 2 millimicrograms (143). Even incubation temperature was observed to be important in the microbiological assay of riboflavin since it affects acid production (144).

*Interrelationships.*—Liver slices from riboflavin-deficient rats were unable to inactivate estradiol under conditions in which slices from controls on the same diets, but receiving adequate amounts of the vitamin, possessed this activity. The loss of inactivating ability paralleled the decrease in riboflavin content of the liver (132, 133). Rats maintained on a low protein diet were unable to retain normal amounts of riboflavin in the liver. Methionine tended to counteract the decrease in the riboflavin concentrations. Supplementation with methionine also maintained the ability of the liver to inactivate estradiol (145).

*Effect on cornea.*—The riboflavin of the cornea was found to be largely concentrated in the epithelium and was promptly influenced by the level of riboflavin intake. Light, either visible or ultraviolet, had no demonstrable effect on the concentration of corneal riboflavin (146). Abnormal vascularization of the cornea has been overcome by treatment with riboflavin (147). However, a uniform peripheral corneal vascularization was found not to be a safe basis for a diagnosis of riboflavin deficiency existing at the time of examination. Such a lesion was observed to be due in some instances to riboflavin deficiency



which occurred previous to the examination. The lesion has also been found to be due to some cause other than a lack of riboflavin (148, 149).

*Effects of deficiency.*—Riboflavin deficiency, produced in the monkey, was characterized by a striking "freckled dermatitis" and an anemia. In the anemia, hemoglobin levels and red blood cell counts were reduced to half normal or less. White blood cell counts were also markedly reduced. In contrast to thiamine-deficient animals, the riboflavin-deficient monkey continued to eat and anorexia was never observed even in advanced deficiency (150). Riboflavin deficiency in swine resulted in growth impairment, rough, dry, and thin hair coats, a mottled erythematous eruption together with scaling and ulceration of the skin, lens opacities, normocytic anemia, and abnormal gait (151). Congenital malformations in rats, most conspicuous in the skeleton, were prevented when the maternal diet was supplemented by riboflavin (152).

In mice, riboflavin deficiency seemed to increase their resistance to the Lansing strain of poliomyelitis virus (153). Riboflavin-deficient animals were also shown to be much more highly susceptible to *Salmonella* infections than were control mice of the same stock (154). Riboflavin deficiency increased mortality in *Plasmodium lophurae* infections in chicks (155).

*Requirements.*—In a study of the riboflavin requirements of children of pre-school age it was concluded that a riboflavin intake of approximately 0.50 mg. per 1,000 Cal. met the needs of the subjects (123). The excretion of riboflavin in the urine of boys between the ages of ten and sixteen placed on a riboflavin-low diet, after a preliminary drop, tended to remain constant at a value about two times that of the riboflavin intake. The fecal excretion remained unaffected at a level of five to six times the intake, a phenomenon which was attributed to synthesis of riboflavin by the intestinal bacteria. An attempt to inhibit the biosynthesis of riboflavin by administering succinylsulfathiazole failed. The conclusion was drawn that riboflavin may not be a dietary essential under all conditions (156).

In studying the physiological and biochemical functions in normal young men on a diet restricted in riboflavin it was shown that work performance and the bodily responses to work were essentially unaffected by dietary alterations. Normal young men, therefore, appeared to suffer no physiological handicap from subsistence for at least five months on a diet providing 0.31 mg. of riboflavin per 1,000 Cal. This

represented about one third the National Research Council recommendation (157). From a study of the riboflavin content of diets served in the Royal Air Force messes it was concluded that the average riboflavin requirement of adults does not exceed 2 mg. daily since no sign of deficiency of riboflavin existed in the personnel receiving these diets (158). On a diet containing an average of 1.6 mg. of riboflavin per day men in a camp in Northern Africa were observed to be free from stomatitis. Stomatitis developed, however, about two months after the daily riboflavin intake was reduced to 1 mg. per day and it was not abolished by an intake of 1.28 mg. per day (159). In a study on the riboflavin requirements of man, the saturation tests were seldom found to give more information than can be obtained by analysis of a fasting morning specimen. Evidence has been presented that the riboflavin per ml. of urine is a more constant value than others which have been used in expressing riboflavin excretion (160).

On the basis of riboflavin excreted in the urine evidence has been presented that riboflavin is a dietary essential for the horse. A daily intake of 44  $\mu$ g. of riboflavin per kilogram of body weight per day adequately satisfied the requirements of horses under the experimental conditions (161).

Riboflavin has been shown to be important in turkey poult nutrition. The turkey poult required approximately 270  $\mu$ g. of riboflavin per 100 gm. of ration for normal development during the first six weeks of life (162).

*Miscellaneous.*—Riboflavin has been observed to play an important role in the economy of food utilization, both for growth and for maintenance. Although moderate inanition occurs, the most logical explanation for the poor food economy in riboflavin deficiency appeared to be that the intermediate products of metabolism are wasted through incomplete combustion (163). In evaluating the efficiency of utilization of increasing doses of riboflavin it was impossible to evaluate the smaller doses because of bacterial synthesis. However, massive doses of riboflavin were much more economically utilized than massive intakes of thiamine (128).

The riboflavin requirement of the rat was shown to be affected by the composition of the diet, especially in respect to its carbohydrate content. Dextrin and corn starch increased the amounts of available riboflavin synthesized in the intestines by providing intestinal microorganisms with favorable conditions whereas sucrose did not. Since fat aggravated the production of spastic paralysis in riboflavin-deficient

rats a role of riboflavin in nerve metabolism was suggested (164). Riboflavin has been shown to increase the resistance of the organism to low oxygen concentration (165).

#### NICOTINIC ACID

*Chemistry.*—Analysis of wheat bran and certain other natural materials for nicotinic acid has revealed the presence therein of a water-soluble substance which appears to be an unidentified derivative or precursor of this vitamin. Studies dealing with this precursor suggested that it contained the nicotinyl radical attached to a substituent, bearing functional groups which render the entire molecule acidic and water-soluble. The complex molecule was readily hydrolyzable to form nicotinic acid (166). The precursor appeared to be utilized by the dog as a source of nicotinic acid. Several sugar derivatives of nicotinamide did not resemble the precursor in their action on *Lactobacillus arabinosus*. On the other hand, several simple esters of nicotinic acid exhibited a type of biological activity similar to that of the precursor. In making either microbiological or chemical assays, autoclaving the sample with 1 *N* sodium hydroxide, 1 *N* hydrochloric acid, or 1 *N* sulfuric acid so as to completely hydrolyze the precursor was found to be necessary (167).

Certain amino acids and nonnitrogenous dibasic acids have been found capable of substituting for glutamic acid which had previously been shown capable of replacing nicotinamide as a growth factor for certain microorganisms. The ammonium salts of a few dibasic acids produced small amounts of nicotinamide activity although their sodium salts were completely inactive (168). A new chemical-catalytic procedure has been reported for the oxidation of nicotine,  $\beta$ -picoline, and quinoline to nicotinic acid (169). Coramine which is inactive for *L. arabinosus* was rendered as active as nicotinic acid by autoclaving one hour with 0.5 *N* sodium hydroxide (170).

*Related substances.*—The urinary level of  $F_2$  was found to be correlated with dietary and clinical evidence concerning the nicotinic acid stores of patients, although there was some variability in this respect. The variability of  $F_2$  in the urine seemed to be due to the presence of other intermediaries of nicotinic acid metabolism besides  $F_2$  (171). The fact that *N*-methyl nicotinamide chloride was found to be effective in preventing as well as curing blacktongue in dogs, if not given too late, suggested that the  $F_2$  nucleus (*N*-methyl pyridine  $\beta$ -carboxy-) is the active antipellagra vitamin (172). The isolation and properties

of  $F_2$  and an improved procedure for its determination have been described (173, 174).

A direct correlation was observed between the nicotinic acid and coenzyme I levels in muscle and the nicotinic acid content of the diet. A similar correlation was noted between the dietary nicotinic acid and liver content, but the coenzyme I levels increased only slightly in the group on the highest nicotinic acid supplement (175).

*Assay.*—Most chemical methods for the determination of nicotinic acid involve reaction with cyanogen bromide and a primary or secondary aromatic amine. Difficulties in connection with the color reaction were eliminated in a method reported for the colorimetric determination of nicotinic acid (176). Methods have also been described for the estimation of nicotinamide methochloride and nicotinic acid in urine (72, 177, 178).

*Metabolism.*—Total daily urinary elimination of nicotinamide methochloride by man has been found to vary considerably. The height to which nicotinamide methochloride elimination rises appeared to be determined not only by the intake of nicotinamide and related compounds, but also by their need in the body, the presence of sufficient methyl-donors, and the efficiency of the methylating-mechanism (179). A marked reduction in urinary output of nicotinamide methochloride in man occurred during administration of sulfaguanidine and succinylsulfathiazole. The most obvious interpretation was to assume the synthesis and release of the vitamin by intestinal bacteria, which action is impaired by the bacteriostatic action of the drug (180). Differences in the manner in which nicotinic acid and nicotinamide are metabolized in the human organism have been described (181).

*Animal experiments.*—The ingestion of large quantities of nicotinamide by young rabbits and guinea pigs did not impair their growth. Since neither of these species excreted  $N'$ -methylnicotinamide during nicotinamide feeding, it was concluded that the toxic effects of nicotinamide in the rat are due entirely to the synthesis of  $N'$ -methylnicotinamide and consequent depletion of available methionine (182).

Dogs fed diets deficient in nicotinic acid have been observed to develop a profound macrocytic anemia. A sharp reticulocyte response and subsequent elevation of the red cell count and hemoglobin concentration were noted after the administration of nicotinic acid and nicotinamide (183).

In the case of induced necrotic enteritis in swine, nicotinic acid and a principle, or principles, present in liver, but not in sufficient

quantities in yeast, increased gains and shortened the recovery period although the disease was not prevented (184).

Evidence has been presented for the nonessentiality of nicotinic acid in the diet of the horse. Balance studies showed that the output of nicotinic acid in the feces and urine was considerably greater than the nicotinic acid intake on the nicotinic acid low diet. This suggested that the vitamin was synthesized in the body of the horse (185).

*Medical application.*—Since the urinary excretion of nicotinic acid and its derivatives was lower in patients with pellagra and vitamin B complex deficiency than in normal patients and since a large test dose of nicotinamide magnified this difference in excretion, the measurement of the quantity of derivatives of nicotinic acid excreted in six hours following the oral administration of 300 mg. of nicotinamide was suggested as a useful procedure in evaluating the nutritional status of a person with regard to nicotinic acid (186).

The successful treatment of a case of post-traumatic confusional state with massive doses of nicotinic acid, was explained on the basis that the vitamin as a vasodilator caused increased flow of blood and thus improved the cerebral metabolism. It was suggested that administration of nicotinic acid may shorten the course of some traumatic psychoses and possibly help to prevent chronic disability (187). It was also suggested that the occasional, unexplainable general cases of mental confusion or stupor observed in middle-aged or elderly persons, might be due to nicotinic acid deficiency (188).

#### PYRIDOXINE AND RELATED COMPOUNDS

*Stability.*—Pyridoxine has been found to be unstable when irradiated in aqueous solutions at pH 6.8 or above, but was almost unaffected at pH 1.0. It also was unstable in nitric acid solutions at 100° C. or to treatment with permanganate and hydrogen peroxide even at room temperature. On the other hand, the vitamin was stable when autoclaved at fifteen pounds pressure in strong acid or alkali (189).

*Assay.*—Studies have been conducted with pure systems in order to develop a chemical method for the determination of pyridoxine based upon its coupling reaction with 2,6-dichloroquinonechloroimide in a one-phase system (190). The method was applied to the determination of pyridoxine in biological materials and pharmaceutical products. In making the application, evidence was obtained which

showed that compounds other than pyridoxine, possessing the biological activity of B<sub>6</sub>, are found in natural materials (191).

A method for determining pyridoxine using *Neurospora sitophila* was found to give values of the same order of magnitude as those determined by the rat assay, but it was subject to such variations that it could not be recommended for routine use. In the method of Landy & Dicken, *L. casei* was found to respond both to vitamin B<sub>6</sub> and pseudopyridoxine and probably also to unknown nutrilites. This method therefore could not be regarded as a reliable measure of the pyridoxine value of biological materials. Good agreement, however, was found upon making a direct comparison of the yeast and rat methods for determining pyridoxine (192).

*Animal experiments.*—A study has been made of the relation of the protein level to the acrodynia which develops in rats maintained on pyridoxine-deficient diets. Acrodynia appeared at an earlier date and was more severe in the rats that received diets high in protein than in those fed diets low in protein (193).

Experimental work has been conducted on the relationship between the convulsive seizures associated with pyridoxine deficiency in the rat and those which regularly appear after giving standard auditory tests. No level of pyridoxine was found to be sufficient to afford continued protection from sound-induced seizures, but with higher levels the seizures were both delayed and less severe (194).

Tests have been made to determine the mechanism by which anemia, due to pyridoxine deficiency, is produced in swine by ascertaining the extent of blood destruction and by seeking the cause of the elevated serum iron. When the dietary intakes of iron in pyridoxine-deficient animals were restricted, hemosiderosis of the tissues and ferremia were prevented, but the serum iron was maintained at the low level seen in iron deficiency. Limiting the iron intake did not alter the ataxia and other symptoms in pyridoxine deficiency (195).

In pyridoxine deficiency in swine the excretion of "kynurenine" and xanthurenic acid has been observed to be above normal and positively correlated with tryptophane intake. When an adequate amount of pyridoxine was given, the excretion of these two substances ceased. Another pigment, red in acidified urine, and thought to be urōrosein, was also found to be related to intake of both pyridoxine and tryptophane (196).

Pyridoxine, when administered in doses several times greater than required for the nutrition of Pekin ducklings, has been found to in-

hibit the activity of minimal effective doses of quinine and atabrine against *Plasmodium lophurae* infections in this species (197).

*Medical applications.*—Agranulocytic angina precipitated in one case by sulfathiazole, in another by aspirin, and in a third by thiouracil has been relieved by administration of pyridoxine (198). Complete or considerable relief from the nausea and vomiting of pregnancy has been obtained in patients by administration of pyridoxine. No toxic manifestations were noticed (199). A combination of pyridoxine and thiamine was observed to be superior to pyridoxine alone in relieving the nausea and vomiting of pregnancy. The results were much better in first pregnancies than in later pregnancies, whether pyridoxine was given alone or combined with thiamine (200).

*Related compounds.*—An investigation has revealed that mixtures which heighten growth-promoting activity for lactic acid bacteria are formed from pyridoxine by procedures causing possible amination and those causing partial oxidation. The amine of 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine, and the aldehyde, 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine were synthesized. These compounds were found to be active for lactic acid bacteria and were named pyridoxal and pyridoxamine respectively (201 to 204).

As a consequence of studies which showed that hydrogen peroxide treated pyridoxine and the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine stimulated the growth of *Lactobacillus casei*, studies were undertaken to determine if these products were required by the chick. The results showed that they significantly increased growth and the hemoglobin content of the blood (205).

The isolation of 2-methyl-3-hydroxy-4-carboxy-5-hydroxy-methylpyridine from urine after ingestion of pyridoxine has been reported. Heating with mineral acid converted this compound to the lactone. The lactone was also prepared from pyridoxine by oxidation with permanganate. Both the acid and the lactone were found to fluoresce, the latter much more intensely than the former. A fluorometric method for the quantitative determination of either compound was developed (206).

*Tyrosine decarboxylation.*—In studies on tyrosine decarboxylation by *Streptococcus faecalis*, two to five times the concentration of pyridoxine was found necessary for maximum decarboxylation activity as was necessary for maximum growth and maximum rate of glucose fermentation (207,208). A derivative of pyridoxine present in acid autoclaved yeast extract and in pyridoxine solutions, treated with

cystine or with dilute hydrogen peroxide, was found to function in the decarboxylation of tyrosine (209). Pyridoxal was also found to stimulate the tyrosine decarboxylase system of *Streptococcus faecalis* R, whereas pyridoxamine and the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine did not (205, 210).

An enzyme preparation from *Streptococcus faecalis* R grown in a medium deficient in pseudopyridoxine has been found to stimulate decarboxylation of tyrosine very slowly. Stimulation was slightly improved by the addition of pyridoxal, but was markedly increased by pyridoxal and adenosinetriphosphate. Adenosinetriphosphate alone had little effect or was slightly inhibitory (211).

#### PANTOTHENIC ACID

*Assay.*—A microbiological assay for pantothenic acid using *Lactobacillus arabinosus* has been found to compare favorably with assay methods previously described (212). A yeast growth method for the determination of pantothenic acid has also been described (213). A method of assay for pantothenic acid using young rats was developed on the basis that wheat germ contains adequate amounts of one or more unidentified factors required by the rat, but is comparatively deficient in pantothenic acid. The increase in growth rate was proportional to the logarithm of the amount of calcium pantothenate fed (214).

*Animal experiments.*—The first manifestations of pantothenic acid deficiency in puppies were observed to be an erratic, lowered appetite and a decrease in urinary excretion of the vitamin. Growth and food intake behaved in a parallel manner. Collapse occurred after two months. The deficiency responded to oral therapy. The only gross pathological changes found were fatty livers. Spasticity was observed in the hindquarters during the last week of the deficiency (215).

Using pantothenic acid containing labelled nitrogen, it was shown that rapid excretion of the heavy isotope follows parenteral administration of the vitamin in deficient mice. Its almost complete absence in various organs indicated that the nitrogen of pantothenic acid is not utilized, nor is pantothenic acid stored in any amount (216). In Swiss mice, those fed a synthetic ration deficient only in calcium pantothenate exhibited a definitely increased resistance to Theiler's encephalomyelitis, but little or none to the Lansing strain of poliomyelitis (217).

Pantothenate-deficient albino rats appeared to be less susceptible



to infection with Type I pneumococcus than litter mates receiving the vitamin (218).

*Miscellaneous.*—Pantothenic acid supported growth in *Staphylococcus aureus* in a tryptophane-free medium in the absence of glucose but not in its presence. The pantothenic acid appeared to mediate the metabolism of glucose leading to or involved in the synthesis of tryptophane. The staphylococcal strains appear, therefore, to be incapable of the synthesis of tryptophane except in the presence of pantothenic acid (219).

Increasing the concentration of calcium pantothenate or sodium pantothenate resulted in an increase in the work output of perfused frog muscles (220).

### CHOLINE

*Chemistry.*—Of some thirty-five different compounds related to choline which were tested for their growth-promoting activity for a Type III pneumococcus grown in a semisynthetic medium, the active compounds were found to contain a N-C-C-OH or N-C-C-C-OH linkage. Substitution of, or through, the hydroxyl group resulted in complete inactivation of the molecule. Of all the naturally occurring compounds tested, only ethanolamine was able to support the growth of this pneumococcus in the absence of choline (221).

*Assay.*—A modification of the standard reineckate method, taking advantage of the increased solubility of ammonium reineckate in 1.2 N hydrochloric acid, has been reported for the measurement of the choline content of phospholipids (222). Choline-containing phospholipids were separated from noncholine-containing phospholipids just as completely by adsorbing from methanol solutions as by first adsorbing from petroleum ether solutions and then eluting with methanol (223). A modification of the microbiological method of Horowitz & Beadle using *Neurospora* for the determination of choline has been developed which satisfactorily measures free choline in animal tissue. For the determination of total choline the values obtained by the microbiological method agreed very well with those obtained by the chemical method (224). The microbiological method was also adapted to the determination of free choline in plasma and urine, as well as for the determination of total choline in blood. The free choline in blood occurred chiefly in the plasma with only a relatively small amount in the cells. The choline in urine was found to exist entirely in the free state (225).

*Relation to phospholipid formation.*—Measurements of recovery of radioactive phosphorus from tissue phospholipids showed that choline deficiency decreased phospholipid turnover (226). In weanling rats deficient in choline lower values for lecithin content of the liver were obtained. Fat infiltration of the liver was prevented to a considerable extent by the administration of choline. The action of choline was not duplicated by various choline precursors, although ethanolamine, alone or together with methionine, was effective to a certain extent (227). In more mature rats the changes in liver lipids induced by choline-low experimental diets were not reversed by the administration of choline after seven days of deficiency. On the other hand, when choline supplementation was initiated immediately, a definite effect on liver phospholipids was demonstrated in two-month-old rats as in weanling rats. This reaction was explained on the basis of depletion of the tissue reserves of an unknown factor (228).

With the aid of isotopic ( $N^{15}$ ) choline, the rate at which this constituent of the phosphatides was replaced in rats was determined, both on an adequate and on an alipotropic diet. The effect of choline deprivation was to retard markedly the rate of incorporation of new choline into the phosphatides of the body without altering the quantity of choline in the phosphatides (229). Deprivation of choline caused kidney abnormalities in young rats with a reduction in the actual amount of phospholipid in the kidneys and livers, as well as a decrease in the ratio of phospholipid to total lipid. There was also a decrease in the amount of choline, although the ratio of choline to phospholipid was practically unchanged. The hypothesis was advanced that kidney damage was due to a decreased formation of phospholipid (230).

*Relationships with methionine and betaine.*—Choline and methionine exerted an interchangeable supplementary action in a chick ration consisting largely of corn and soybean oil meal (231). Betaine and methionine have been shown to be effective in preventing perosis and promoting growth in chicks when added to a simplified diet containing some natural ingredients, but ineffective when added to a purified diet of higher methionine content. In contrast, choline prevented perosis and promoted growth on both diets. The results indicated that choline is required as such for the prevention of perosis and for growth and that betaine and methionine in the presence of an unidentified factor enabled the chicks to synthesize choline (232). Betaine and methionine have been reported as being capable of assuming a certain portion of the functions of choline in the chick with arsenocholine capable of

assuming the remainder of the choline functions. Hence, the effect of these together was practically a complete substitute for choline. It was pointed out in this connection that the practical dietary choline requirements may be modified by the adequacy or deficiency of betaine and methionine (233).

*Anemia.*—A depression of the normal erythrocyte number was produced in dogs by giving choline hydrochloride. Atropine fed simultaneously nullified the effect of choline. The results were explained by assuming that choline depressed erythropoiesis by increasing the blood flow and oxygen supply to the bone marrow through its vasodilator action (234). Daily supplements of lard and choline to the diet of dogs has been found to result in a rapid reduction of red blood cell count and percentage hemoglobin. Discontinuing fat feeding alone or of both fat and choline allowed rapid return to normal. The results were interpreted as indicating that fat caused hemolysis, although the erythropoiesis kept pace with red cell destruction if no choline was fed, so that anemia failed to develop. If choline was fed simultaneously, however, erythropoiesis was hampered and anemia developed (235). A hyperchromic anemia was produced by choline in dogs which responded not only to atropine feeding but also to injections of U.S.P. liver preparation and to the feeding of U.S.P. stomach preparation (236).

*Choline toxicity.*—Choline hydrochloride has been claimed not to be highly toxic. On the other hand, lethal doses have been reported for several species. This discrepancy has been reconciled in part by the results of an investigation which showed that the amount of choline hydrochloride necessary to kill the average rat is greater, the more dilute the solution injected intraperitoneally. The symptoms of poisoning were changes in respiration followed by trembling, convulsive movements, salivation, hemorrhage around the eyes, cyanosis, respiratory paralysis, and death (237). A case of choline poisoning in cattle was reported due to feed containing a large amount of free choline. After first calving the uterus did not contract, but remained open and atonic for a considerable time, thus forming a source of secondary infections. This was not influenced by usual medical treatment and resulted in inability often to conceive and in abortion. The findings point to the fact that free choline can act as a toxic agent when fed over long periods of time (238).

*Choline deficiency.*—Fatal deficiency of choline has been produced in less than three hours in dogs. The deficiency was characterized by

severe fatty infiltration of the liver. A rise in blood plasma phosphatase, an impairment in bromsulfalein elimination, and a fall in blood plasma cholesterol and cholesterol esters were observed. In severe deficiency there was an increase in prothrombin time and a decrease in blood hemoglobin, hematocrit, and plasma proteins. The total cholesterol concentration of the liver was unchanged although total lipids were increased from three to four times (239).

The inclusion of atabrine in low choline diets at a level of 65 mg. per 100 gm. of ration almost completely prevented hemorrhagic kidneys in the rat. A level of 40 mg. per 100 grams prevented death but allowed some kidney damage to develop. No lipotropic action was observed at the various levels fed (240). The creatine content of the gastrocnemius muscle of young male rats on diets varying in choline content was determined. No lowering of the creatine content was revealed in the rats fed a diet deficient in choline for a period of three weeks (241).

*Requirements.*—Strain differences in the choline requirements of rats have been demonstrated. One strain of rats required approximately 4 to 6 mg. per day and another strain of rats maintained on the same diet required about 10 mg. per day. It was also observed that the females tended to require less than males. The strain differences in requirement were present throughout three generations of offspring of the original parent stock (242).

An increased choline requirement for rats in hot environments has been reported. Diets containing 0.75 gm. of choline per kilo of food gave optimal growth at 68° F., while for the same growth rate at 90 to 91° F., 5 gm. of choline were needed. The requirement was not increased until the rats had been kept for at least two weeks in the hot environment (127).

*Medical application.*—A number of patients with decompensated portal cirrhosis of the liver showed distinct improvement within a week after the start of choline therapy. In view of this finding, the use of choline as an adjuvant to dietary therapy of cirrhosis of the liver, particularly of the fatty, alcoholic type, seemed to be justified (243).

*Miscellaneous.*—In contrast to the observation that cholinergic nerves are stimulated by salts of choline, acetylcholine, acetyl- $\beta$ -methyl-choline, and carbamyl choline, it has been found that di-n-butyl carbamyl choline chloride has a depressing action on cholinergic nerves (244).

## BIOTIN

*Chemistry.*—A detailed description of the synthesis of biotin has been presented. The starting materials for this synthesis were *l*-cystine, chloroacetic acid, and glutaric acid. The relationships of the various isomeric forms of biotin and the side reactions encountered were also described (245, 246, 247).

Phosgene has been found to convert diaminopelargonic acid back to desthiobiotin. The diaminopelargonic acid preparation was observed to exhibit 10 per cent of the activity of desthiobiotin (248). The total synthesis of 2,3,4,5-tetrahydrobiotin using as starting materials trimethylene chlorobromide and ethyl malonate has been reported (249).

*Assay.*—The determination of biotin by means of *Lactobacillus arabinosus* has given results which are comparable with those obtained by means of procedures using *Lactobacillus casei*. In developing the method the existence of a water soluble avidin-combinable form of biotin which is available for *L. casei* but not for *L. arabinosus* was discovered. However, by autoclaving natural materials with 6 *N* sulfuric acid concurrent values were obtained with both species (250).

A study of the factors affecting the determination of biotin by means of *L. casei* has shown that it must be present in the form of the free acid. Under various conditions, the activity of biotin methyl ester was found to range from 0 to 100 per cent. Variations in activity were also caused by the casein hydrolyzate, by the presence of copper salts in low concentration, and by the use of hydrogen peroxide-treated norite yeast filtrate in place of vitamin B<sub>6</sub>. A medium containing yeast filtrate, however, was found to be better than a synthetic medium (251).

*Metabolism.*—Biotin-deficient *Saccharomyces cerevisiae* have been found to respire and ferment at rates much less than those of normal biotin-rich yeast. Upon the addition of biotin, in the presence of ammonia these metabolic rates rose gradually. With yeast only partially deficient in biotin, the addition of the vitamin sometimes caused an immediate increase in fermentation rate, even without the addition of ammonia. Unless biotin was present, no ammonia was taken up by biotin-deficient yeast (252).

In *in vitro* studies on the respiratory metabolism of tissues from biotin-deficient animals, small amounts of biotin, added to slices of biotin-deficient rat liver respiring in Ringer-bicarbonate solution containing lactate as substrate, have been found usually to cause a slight

rise in both the oxygen consumption and the respiratory quotient. In every case, the presence of added biotin was associated with a marked change in a negative direction in the aerobic  $Q_G$  value, the so-called "aerobic glycolysis." When pyruvate was used, the effects were quite similar to those described for lactate (253).

*Interrelationships.*—Biotin sulfone, derived from biotin by oxidation of the sulfur to the corresponding sulfone, has been reported to stimulate the growth of yeast. The yeast growth, however, was not increased above a relatively low maximum even with large amounts of sulfone. The sulfone combined with avidin which indicated that the oxidized form of the sulfur does not interfere with the interaction of biotin and avidin (254).

During the past year the avidin activity of a number of avidin preparations has been found to be proportional to lysozyme activity, and the lysozyme activity of a sample of egg white lysozyme was found to parallel its avidin activity. The addition of biotin was observed to increase greatly the activity of lysozyme. The results of these studies suggest strongly that the lysozyme activity of avidin concentrates is due to the avidin-biotin complex (255, 256).

In studies on the growth-promoting and antibiotin activities for *S. cerevisiae* and *L. casei* of certain compounds structurally related to biotin, compounds which had a urea ring and a carboxylic acid side chain of five or six carbon atoms were able to combine with avidin, whereas all compounds which lack the cyclic urea structure were unable to do this. The side chain also seemed essential. Biotin sulfone and two analogues of desthiobiotin, when added to *S. cerevisiae* or *L. casei* cultures in the presence of avidin-bound biotin, were observed to be capable of displacing some of the vitamin which then became available for the growth of the organism (257).

Previous findings that *L. casei* does not grow when desthiobiotin replaces the biotin of the media whereas *Saccharomyces cerevisiae* strain 139 grows readily have been extended in experimental work with forty-five biotin-requiring organisms. Desthiobiotin was found to replace biotin for twenty-five strains of *Saccharomyces cerevisiae*, but failed to do this in the case of five other microorganisms. In two of these five instances desthiobiotin did not act as an antibiotin in the presence of an exogenous supply of biotin (258).

A mixture of biotin and desthiobiotin has shown an additive effect on the growth of *Saccharomyces cerevisiae*. Mixtures of desthiobiotin at low concentration and biotin promoted growth with *L. casei* which

was identical with that obtained with biotin alone. At higher concentration desthiobiotin possessed a definite antibiotin effect toward *L. casei*. This inhibition of growth was completely reversed by increasing the biotin concentration to still greater amounts. Results were also obtained which indicated that desthiobiotin disappears from the incubating yeast cultures and is replaced by an equivalent amount of a substance possessing growth-promoting powers for *L. casei* (259).

*Animal experiments.*—Biotin-deficiency, induced in weanling rats by the feeding of a purified diet containing succinylsulfathiazole, has been reported to be aggravated by superimposing a deficiency of pantothenic acid. The feeding of biotin protected against these changes and, in addition, appeared to lessen the severity of the syndrome associated with a lack of pantothenic acid. When calcium pantothenate was fed prophylactically, signs of a mild degree of biotin depletion only were observed (260). Evidence has been presented that progressive ascending paralysis in dogs due to a deficiency of a vitamin B complex factor found in yeast responds promptly to synthetic biotin therapy (261). Biotin has been found to be required by mice maintained on a completely artificial diet. The deficiencies were more acute when succinylthiazole was included in the diet (262).

Further evidence has been obtained of the effects of biotin on infection by injecting both biotin-deficient and normal chicks with *Plasmodium lophurae*. The parasite counts were significantly higher in the biotin-deficient chicks than they were in the normal ones (263).

*Miscellaneous.*—A study of the requirements for biotin in the growth of pneumococci has shown that biotin is an essential growth factor for all thirty-three cultures, including twenty-six types, of pneumococci tested (264). The synthesis of biotin by a sulfonamide-sensitive and a sulfonamide-resistant strain of *Escherichia coli* was not greatly affected by the presence of sulfanilamide in the growth medium (265). The biotin content of sarcomas grown in tissue culture has been found to be about the same as that of sarcomas produced *in vivo* (266). An investigation has shown that the avidin-uncombining forms of biotin appear not to be generally distributed in nature but are found in urine only (267).

#### FOLIC ACID, LACTOBACILLUS CASEI FACTOR, STREPTOCOCCUS LACTIS R FACTOR AND VITAMIN B<sub>6</sub>

*Chemistry.*—A procedure has been described whereby folic acid was concentrated from spinach to such an extent that the product was

about 137,000 times as active as a standard liver extract (Wilson's liver fraction "B") (268). In quantitative studies of charcoal adsorption of folic acid from crude preparations and from concentrates, elution from crude preparations was found to be much easier than from relatively pure solutions (269). On the basis of analysis of some of the concentrates, together with information on molecular weight, the empirical formula was indicated to be  $C_{15}H_{15}O_8N_5$ . The absence of a sugar or a polyhydroxy group and the probable presence of a xanthopterin-like structural unit was indicated by the results (270). The absorption spectra of folic acid preparations were also found to resemble that of xanthopterin. Evidence was given indicating that the difficulties of purification of many folic acid concentrates are probably due to impurities consisting of inactivated folic acid with physical properties only slightly changed (271).

*Assay.*—The method of Mitchell & Snell has been found satisfactory for determining the folic acid content of liver preparations, but inadequate in several essential factors when other materials were assayed. In order to facilitate the use of *Streptococcus lactis* R in assay work, the optimum amounts of the various factors for the growth of this organism on a casein-hydrolysate synthetic medium were determined, the results of which were used to build a new basal medium (272).

In assaying typical fractions from liver and yeast autoclaving the samples with either acid or alkali has been found to cause an optimal increase in the *Streptococcus lactis* activity. In each case, the *Lactobacillus casei* activity corresponded well with the *Streptococcus lactis* activity (273). In the determination of folic acid by *Lactobacillus casei*, duplicate tubes have been found to produce widely varying amounts of acid. These variations appeared to be due to the extraction of folic acid from the cotton plugs (274).

The results of experimental work on thymine and its nucleoside, thymidine, have shown that these compounds may be substituted for folic acid in the growth of *Streptococcus lactis* R and related lactic acid streptococci. In the case of folic acid-requiring lactobacilli, however, replacement of folic acid by thymine was only partially effective (275).

*Differences in activity.*—In confirmation of the point of view that the *L. casei* factor is in reality a group of substances with a common basic chemical structure, the isolation in crystalline form of a new compound which is highly active for *L. casei* and only slightly active



for *S. lactis* R has been reported. The new compound was also found to be active in the nutrition of the chick (276).

A study of the requirements of a number of lactic acid bacteria for *S. lactis* R factor and folic acid has revealed that some can develop with either the *S. lactis* R factor or folic acid. Other lactic acid bacteria, primarily lactobacilli, were unable to utilize *S. lactis* R factor and required folic acid for growth. The organisms which developed with the *S. lactis* R factor were found in every instance to form folic acid (277).

*Vitamin M and folic acid.*—Results have been presented which showed that the distribution of folic acid as determined by means of *Streptococcus lactis* R was different from that of vitamin M which prevents nutritional cytopenia in the monkey (278). On the other hand, evidence has also been presented which indicated that vitamin M may be identical with folic acid. The high value for the folic acid content of yeast produced by incubation with liver was in much better agreement with the known high vitamin M content of this yeast than was the value for preformed folic acid (279). Also in experiments in which liver extract and yeast extract were used as sources of the factor antagonistic to the leucopenic effect of succinylsulfathiazole in rats, evidence was obtained that the rat antisuccinylsulfathiazole factor is the same or very similar to vitamin M. Both factors appeared to consist of substances which either stimulate *S. lactis* R or which may be enzymatically converted to *S. lactis* R-stimulating substances (280).

*Xanthopterin and folic acid.*—The finding that xanthopterin is able to promote the formation of folic acid by fresh rat liver *in vitro* has been confirmed. Also yeast which has a relatively low preformed folic acid content was found to be rich in the substances which give rise to folic acid when incubated with fresh liver (279). A relatively stable enzyme preparation which can be used in place of fresh liver in converting inactive substances to folic acid has been prepared from rat liver. The purified enzyme preparation differed from liver brei in that the former was incapable of bringing about the production of *S. lactis* R-stimulating factor from xanthopterin (281).

Differing from the effect obtained *in vitro* with liver slices, the addition of xanthopterin to cultures of *Aerobacter aerogenes* significantly decreased the synthesis of folic acid. The folic acid produced by *Aerobacter aerogenes* was more potent for *L. casei* than for *S. lactis* (282).

*Antianemic effects.*—Continued work on the role of crystalline

vitamin B<sub>6</sub> on hematopoiesis in the chick has shown that a deficiency of the vitamin causes retarded growth, a marked decrease in the hematocrit, the hemoglobin, and the red blood cell count, and, in addition, a reduction in the leucocytes and thrombocytes. This syndrome was prevented by fortifying the basal diet with crystalline vitamin B<sub>6</sub> (283). Evidence was also obtained which showed that when the vitamin was given parenterally, it had the same biological effect as when given orally (284).

Yeast and certain yeast extracts have been found highly active in vitamin B<sub>6</sub> as measured by the anemic chick, but had little potency in stimulating the growth of *L. casei*. Concentrates of the chick anti-anemic factor from yeast become highly active in microbiological growth effect following enzymatic digestion. A pure crystalline compound was isolated from yeast which had the same biological activity and physical properties as vitamin B<sub>6</sub> from liver (285).

Crystalline *L. casei* factor has been found to have a preventive and curative effect on severe anemia in rats. The anemia was produced in rats fed a highly purified diet containing sulfasuxidine by subjecting them to hemorrhage (286).

*Animal experiments.*—Evidence has been obtained that an adequate intake of the *L. casei* factor is essential for the normal metabolism of stilbestrol in the chick. Large doses of stilbestrol were found to produce only a slight weight increase in the oviduct of chicks fed a diet deficient in *L. casei* factor, whereas, a large weight increase was observed in the oviducts of control chicks receiving *L. casei* factor supplement either curatively or prophylactically (287).

In view of the exceedingly low folic acid content of milk a study has been made of the value of a suitably supplemented dried whole milk diet for the production of folic acid deficiency in the rat. Assays for folic acid in the tissues of these rats showed that considerably larger amounts of microbiologically active materials were present in the hepatic tissues of animals fed the whole milk diet than were found in the livers of rats fed a highly purified diet containing a comparable amount of folic acid. The inclusion of succinylsulfathiazole in both diets caused a marked reduction in the folic acid content of the liver (288).

The addition of folic acid in the form of a norite eluate to highly purified diets has been observed to improve lactation in three strains of mice reared for several generations on this diet. The growth on these artificial diets, however, was excellent in spite of the folic acid

deficiency, and better than that on the stock diet (289). A folic acid concentrate and crystalline *L. casei* factor have been found to be strong inhibitors of tumor growth in mice. On a weight basis the *L. casei* factor was a many hundredfold stronger inhibitor than previous work had shown inositol to be (290).

The successful substitution of crystalline folic acid for one of the three unknown dietary essentials required by guinea pigs in addition to the known vitamins has been reported. The folic acid replaced one of the factors shown to be present in linseed meal (291).

#### *p*-AMINOBENZOIC ACID

*Assay.*—A microbiological assay for *p*-aminobenzoic acid in which *Clostridium acetobutylicum* S9 is employed as the test organism has been developed. The assay range is 0.3 to 1.5 m.μg. of *p*-aminobenzoic acid per 10 cc. Growth is determined by turbidity at twenty to twenty-four hours. Autoclaving at 75 to 80 pounds for one hour in 5 *N* sodium hydroxide gave maximum *p*-aminobenzoic acid content (292). Other evidence that *Clostridium acetobutylicum* is satisfactory for assays of *p*-aminobenzoic acid was obtained in a study in which turbidity and acid production were found to be correlated with *p*-aminobenzoic acid content (293).

*Sulfonamide resistance.*—The development of sulfonamide-resistant strains of *Staphylococci* have been found to be related quantitatively to the production of *p*-aminobenzoic acid by this species of bacteria. The sulfonamide-resistant strains produced more *p*-aminobenzoic acid than the sulfonamide-sensitive strains (294).

The sulfonamide resistance of *Neisseria gonorrhoeae* has also been observed to be related to synthesis of *p*-aminobenzoic acid. With but few exceptions, patients promptly cured by sulfonamides yielded sulfonamide-sensitive cultures with little ability to synthesize *p*-aminobenzoic acid. Clinically-resistant individuals yielded sulfonamide-resistant cultures with great ability to synthesize *p*-aminobenzoic acid (295).

On the other hand, evidence has been obtained which appears to contradict the claim that resistance to sulfonamides is associated with the formation of *p*-aminobenzoic acid. The growth of *Staphylococci*, normally resistant to sulfonamide, in a medium lacking glucose was inhibited either by sulfanilamide or sulfathiazole, but not in a medium containing glucose. When glucose was replaced by pyruvate, growth was largely inhibited by sulfonamides, in spite of the fact that the

amount of *p*-aminobenzoic acid formed under these conditions was greater than when glucose was present in the medium. *Staphylococcus albus*, which is not dependent on glucose and does not yield a measurable amount of *p*-aminobenzoic acid, was not inhibited by the sulfonamides (296).

The formation of *p*-aminobenzoic acid by a strain of *Staphylococcus aureus* rendered resistant to sulfonamides has been found to depend on the presence simultaneously of glucose or pyruvate and tryptophane. In the presence of glucose with no tryptophane only a small amount of *p*-aminobenzoic acid was formed. Substitution of glucose and pyruvate with glycerol or certain aliphatic acids in the presence and absence of tryptophane did not cause the formation of *p*-aminobenzoic acid (297).

*Antisulfonamide activity.*—*p*-Aminobenzoyl-*l*(+)-glutamic acid when compared with *p*-aminobenzoic acid has been found to possess very little or no antisulfonamide activity with strains of *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Escherichia coli*, and *Clostridium acetobutylicum*. With *Lactobacillus arabinosus*, the antisulfanilamide activity of *p*-aminobenzoic acid was twenty times greater than that of *p*-aminobenzoylglutamic acid (298). Numerous *N*-alkyl *p*-aminobenzoic acid derivatives have failed completely to have an effect upon the antibacterial action of sulfathiazole (299).

*p*-Aminobenzoic acid has been shown not to counteract the effect of sulfapyrazine in inhibiting the growth of *Bacterium tularensense* on a favorable liquid medium, but instead inhibited growth if applied in high concentrations (300). Thirty-five compounds related in structure to *p*-aminobenzoic acid have been studied to determine their effects upon the growth of bacteria. Twelve of the compounds showed bacteriostatic effects which were reversed by *p*-aminobenzoic acid, whereas three compounds behaved like *p*-aminobenzoic acid, although much more weakly. One of the compounds showed bacteriostatic effects at high concentrations and *p*-aminobenzoic action at lower concentrations (301).

*Effect of pH.*—Owing to difficulty in divorcing the effect of pH on sulfonamides from that on *p*-aminobenzoic acid in bacterial inhibition experiments, *Neurospora crassa* has been used as the test organism in studying the action of pH on the effectiveness of the acid alone, because it requires an exogenous supply of this substance. The results showed that the effectiveness of *p*-aminobenzoic acid as the growth factor decreases with the increase in pH (302).

*Formation of yellow pigment.*—The formation of a yellow pigment from *p*-aminobenzoic acid by a special strain of *Mycobacterium tuberculosis* has been found to be brought about by an enzyme which appears to be a specific oxidase (303). Under certain conditions, the formation of the yellow pigment in the presence of *p*-aminobenzoic acid was diminished or inhibited by sulfanilamide (304).

*Medical application.*—In two seasons of experience with typhus fever in Egypt the observation has been made that the administration of *p*-aminobenzoic acid lessened the severity of the disease. The patients receiving *p*-aminobenzoic acid developed few of the complications that normally accompany typhus (305).

Because a deficiency of the acetyl radical has been thought to account for the insufficient production of acetylcholine in myasthenia gravis, the supply of acetyl radical in cases of this character has been investigated. This was done by administering *p*-aminobenzoic acid which may be acetylated by man if taken orally. The results indicated that no serious insufficiency of acetyl radical was present in myasthenia gravis as the proportions of free and acetylated *p*-aminobenzoic acid were substantially the same in both normal individuals and myasthenia gravis patients (306).

*Miscellaneous.*—The injection of *p*-aminobenzoic acid into typhus infected yolk sacs has been found to have rickettsiostatic activity approximately equal to that of penicillin. *p*-Aminobenzoic acid was also found to possess a very effective chemotherapeutic action on murine typhus infection in mice when added to the food in a concentration of 3 per cent (307).

The addition of *p*-aminobenzoic acid to aqueous solutions of *p*-aminobenzenesulfonamide has been found to cancel the retarding effect of the latter on the growth of wheat plantlets. The initial delaying effect of the sulfonamide on the early stages of germination and growth of certain fungi was also eliminated by very small amounts of *p*-aminobenzoic acid (308). Using *p*-aminobenzoic acid in relatively nontoxic concentrations, the inhibitory effect of sulfanilamide upon the growth of oat roots was not completely neutralized (309).

#### INOSITOL

The nutritional significance of inositol has been reviewed (310). A yeast growth method employing *Saccharomyces cerevisiae* as the test organism has been developed for the estimation of inositol in natural products (311). A chemical method has also been developed by

means of which the inositol present in tissues of the rat has been determined (312). Rats fed sulfasuxidine with a specially purified diet were found to maintain life over a period of eight weeks with difficulty. Supplementing the diet with folic acid and biotin allowed a much improved gain in weight but did not prevent alopecia effectively. Additions of inositol, however, in addition to folic acid and biotin, allowed good weight gains and on this regime alopecia occurred in only one out of thirty-six animals (313). In support of previous work, the lipotropic action of choline was shown to be increased in the presence of Mazola oil. The lipotropic action of inositol, on the other hand, was obliterated by the inclusion of Mazola oil. A satisfactory explanation was not at hand, but it was pointed out that choline has a relatively greater lipotropic effect on the "fat" fatty livers than on the "cholesterol" type of fatty livers, whereas with inositol, the reverse is true (314).

#### VITAMIN P

A method has been developed with which to test the vitamin P activity of materials. In this method air is suddenly evacuated from a jar containing mice, and the degree of hemorrhage of the lungs so produced is used as the index of deficiency (315). Using alterations in capillary resistance in man as the index, assessment of the vitamin P potency of certain fruits and vegetables was made. Good sources of the vitamin included grapes, citrus fruits, plums, and prunes (316). The guinea pig capillary-resistance method was also applied to the estimation of vitamin P in some fruits and fruit products. Citrus fruits were the richest source of vitamin P investigated. Little vitamin P activity was lost in the processing of commercial concentrates, but the activity diminished to some extent during storage for several months, even in the refrigerator (317).

#### OTHER WATER-SOLUBLE VITAMINS

Further information has appeared concerning the unidentified vitamins already reported. Some new factors as well have been postulated.

*Poultry.*—Progress towards the separation and isolation of vitamins B<sub>10</sub> and B<sub>11</sub> has been made and additional information on their chemical properties obtained as they exist in impure concentrates (318, 319). Further work has been presented, the results of which confirmed previous reports and showed that neither factor R nor factor S is identical with folic acid or vitamin B<sub>9</sub>. The probable iden-

tity of factor R and vitamin B<sub>11</sub>, however, was indicated (320). An unidentified factor has been shown to be required for reproduction in chickens fed a soybean meal ration. This factor was supplied by meat scraps and as little as 0.2 per cent of Wilson and Company's "Liver fraction C" (321). Practical chick rations containing adequate riboflavin have been shown to require in addition an unidentified growth factor or factors in order to make them satisfactory for feeding. The unidentified factors were demonstrated to be present in liver, milk, condensed fish press water, or ground fish viscera (322, 323).

A severe anemia was developed in pigeons maintained on a purified diet containing the vitamin B complex. The hemoglobin levels were restored to normal by administering extracts of yeast, liver, or rice bran. Evidence was also presented to the effect that a second or weight restoration factor is required by the pigeon (324).

*Other animals.*—In studying the "antistiffness" factor for guinea pigs, the most striking changes occurring during the deficiency were shown to be an increase of inorganic phosphorus and decrease of easily hydrolyzable phosphorus, and excessive calcium deposits in body tissues. The alkaline serum phosphatase in guinea pigs deficient in the "antistiffness" factor was also lower than in nondeficient animals (325, 326). Dogs fed purified diets with synthetic B-complex supplements developed achromotrichia and decreased hair growth in two to eleven months on the diet. Liver fractions or whole dried yeast brought about complete cure (327). Addition of sulfapyridine produced anorexia and weight loss in normal rats and dogs fed a purified diet supplemented with the B vitamins. This condition was counteracted by the inclusion of raw liver in the diet (328). Pigs were seldom observed to grow normally and few survived if they were transferred at two days of age to simplified diets that contained only the known water-soluble vitamins. Water extracts of liver or yeast were found to contain all the unrecognized vitamins required by the pig (329).

*Extrinsic factor in pernicious anemia.*—In testing identified components of the vitamin B complex for extrinsic factor activity, the careful purification required to render crude casein "vitamin free" was also found to be essential for the elimination of the extrinsic factor. The combination of casein so extracted with the pure accessory factors did not reconstitute the extrinsic factor activity of the crude casein. Nevertheless, it appeared reasonable to continue to regard the extrinsic factor as a thermostable component of the vitamin B complex as yet unidentified (330).

## GENERAL

*Human experiments.*—Supplements of thiamine, riboflavin, ascorbic acid, and vitamins A and D have been found to have no significant effect on rate of growth, nutritional status, strength, condition of teeth and gums, or attendance of school children, and no effect upon weight, hemoglobin, blood pressure, absence from work, or output of men working in factories (331). In a more controlled study on school children, no effect has been observed during a period of one year upon the gain in height and weight, hemoglobin, dark adaptation, resting pulse rate, vital capacity, breath-holding or endurance upon receiving approximately one half of the daily requirements of thiamine, ascorbic acid, and vitamins A and D. An improvement was observed, however, in ascorbic acid saturation, and in school behavior as assessed by the teachers, and the incidence and duration of colds were decreased (332).

Absence of rapid deterioration in normal young men doing hard physical work on a restricted intake of vitamins of the B complex, has been reported. This finding was based upon comprehensive clinical examinations and objective tests. Of all the variables measured and observed only the vitamin excretion in the urine reflected the intake (333). Blood pyruvic acid values as well as the blood lactate and pyruvate ratio during fasting and following administration of glucose have been found not to differ from the normal in human subjects living under carefully controlled conditions and ingesting a diet deficient only in the B vitamins even though manifestation of sub-clinical deficiency became evident in two of them (334). Under controlled environmental, postural, and metabolic conditions, human subjects with induced thiamine, riboflavin, or vitamin B complex deficiency have been observed not to show any degree of vasomotor disturbances as evidenced by measurements of skin temperature and by determination of rates of cooling and warming of the body tissues (335).

A severe atypical deficiency disease has been described which is difficult to diagnose and which is likely to be fatal unless proper therapy is applied. Correction was obtained by supplying a high caloric diet rich in proteins, vitamins, and minerals together with synthetic vitamins and extracts of natural vitamin B complex (336).

Nicotinic acid has been found in low concentrations in most samples and dehydroascorbic acid in approximately half of the samples



assayed in a study on the water-soluble vitamins in sweat. Loss in sweat appeared therefore not to be a significant factor in depleting the body stores of water-soluble vitamins (337). In a clinical and biochemical study of cow's milk and honey with added thiamine and ascorbic acid as an essentially exclusive diet for human adults, the ascorbic acid and pantothenic acid values of blood plasma were found to be somewhat low, and the urinary excretion of thiamine was below normal (338). The average riboflavin excretion in the urine in paralytic poliomyelitis has been found to be higher than that in normal specimens, and the average biotin content to be higher than that in the urine of patients convalescing from scarlet fever. The values for pantothenic acid were within the normal range (339).

*Rat experiments.*—In a study on the maintenance of adult rats on diets low in certain B vitamins the rats fed the diet free of thiamine lost weight and died within a few weeks. Most of the adults on the other deficient diets survived for many months. Those deprived of nicotinic acid and choline for a year were comparable in appearance, growth, and survival to rats receiving these vitamins (340). Using various purified single food substances as the sole food the observation has been made that thiamine deficiency develops relatively early while the other B factors were not subject to such early depletion (341).

The prolonged administration of a diet partially deficient in the heat stable fractions of the vitamin B complex has been observed to cause the development of abnormal structural changes in the kidneys. Rats fed a more profoundly deficient diet displayed numerous areas of hemorrhagic infiltrations in the cortical and subcapsular regions (342). The observation has been made that the water content of the bodies of rats maintained on a ration low in the vitamin B complex is higher than that in the controls fed *ad libitum*. No difference, however, was revealed in the water content of pair-fed controls. The change in water content, therefore, was not a direct effect of the vitamin B complex supply (343).

A highly purified diet, adequate in those members of the vitamin B complex required for excellent growth in rats, has been observed to result in a marked reduction in the hepatic stores of folic acid and biotin, compared with the amounts of these factors found in the liver of animals maintained on stock rations. The hepatic storage of these factors was further reduced by the incorporation of succinylsulfathiazole in the synthetic diets. The inclusion of succinylsulfathiazole also caused a reduction in the pantothenic acid content of the liver to a

level as low as that produced by diets lacking in pantothenic acid, in spite of the presence in the diet of a previously adequate amount of the vitamin. Administration of crystalline biotin and a concentrate of folic acid caused prompt restoration of the pantothenic acid content of the liver to normal (344).

Evidence has been obtained that in thiamine and possibly in pyridoxine deficiency the resistance of rats to experimentally induced pneumococcal lobar pneumonia is reduced. This effect was not observed in riboflavin or pantothenic acid deficiency. With the exception of thiamine deficiency, all deficiency groups showed a greater resistance to infection than rats fed a normal diet. Inanition had no apparent effect on the resistance of rats (345). In experiments on neuromuscular regeneration, the neuromuscular weakness of regenerating and nondenervated muscles of rats on B-deficient diets has been found to be of the same order as that found in animals subjected to acute inanition or food withdrawal and B supplements (346). Evidence has been obtained that complete or partial deficiencies of riboflavin, pantothenic acid, thiamine, or pyridoxine depressed the thymus weight of female rats below that of the normal controls and caused premature atrophy in some cases, in spite of the fact that all rats received 10 mg. of choline hydrochloride per day (347).

The administration of thiamine and riboflavin has been found to restore the ability of the liver of rats to inactivate estrone and  $\alpha$ -estradiol, whereas the addition of choline chloride, pyridoxine, or calcium pantothenate to the deficient diet failed to affect the deficient hepatic inactivation of these hormones during a deficiency of the vitamins of the B complex (348).

A mixture of the B vitamins has been found to be quite effective in preventing serine toxicity in rats. Of the single vitamins tested, pyridoxine was the most effective (349). The administration of phemerol, a germicidal quaternary ammonium salt, in the drinking water has been found to result in poor growth in rats fed a synthetic diet. This effect was counteracted by the addition of 5 per cent of whole liver powder (350). Young male rats have been observed to increase their intake of thiamine, riboflavin, and pyridoxine greatly after the daily administration of a toxic level of promin. In the presence of the added vitamins the syndrome characteristic of reactions to promin failed to appear (351). Liver or folic acid concentrate plus biotin have been observed to reduce the mortality rate in rats fed for two months on a purified diet containing sulfaguanidine. Under

the influence of this therapy the liver and splenic lesions also tended to disappear (352).

Previous work, showing that the rate of oxygen consumption in the presence of pyruvate by homogenates of liver from pantothenic acid- and biotin-deficient rats is decreased below that of normal rats, has been confirmed in more detailed investigations. However, as a result of studies of rats maintained on other regimens, the mechanism of the effect appears likely to be an indirect one (353). A deficiency of pantothenate, thiamine, pyridoxine, and certain fat-soluble vitamins has been found to cause a decreased excretion of glucuronic acid in rats, while a deficiency of riboflavin resulted in increased excretion (354). Although deficiency of the vitamin B complex has been considered a factor which influences the signs of magnesium deficiency in rats, a study of this problem has shown that no significant interrelationship exists between magnesium and the deficiencies of the vitamin B complex (355).

Rats have been maintained at a high temperature on high and low intakes of the B vitamin complex while the corresponding pair-fed controls were kept at approximately room temperature. At both levels of vitamin intake, the rats at the high temperature gained more weight and retained more nitrogen, fat, and water than the corresponding controls. The concentration of riboflavin, pantothenic acid, and thiamine was highest in the bodies of the group on the high vitamin intake kept at high temperature and lowest in the low vitamin group kept at this temperature. The nicotinic acid of the tissues remained uninfluenced either by level of intake or temperature (356). No significant differences in content of known B vitamins between the tissues of rats in cold and warm environments have been demonstrated. Temperature is therefore not an important factor in causing variation in the B vitamin content of tissues (357).

The observation has been made that when a polyneuritic rat, restricted to low food intake, is given a daily injection of thiamine, it begins to run frantically, as a consequence of the replacement of the specific thiamine hunger by hunger for bulk food. Riboflavin deprivation and readministration acted in the same manner although not as promptly or as markedly. In view of the results it was concluded that increased running is a basic response to nutritional need and that it is stimulated by the lack of any specific indispensable component of the diet (358).

*Other animal experiments.*—The addition of urea along with read-

ily soluble carbohydrates has been found to increase markedly the synthesis of riboflavin, nicotinic acid, biotin, and pantothenic acid in the rumen of cattle, while the addition of readily available carbohydrate alone had only a small effect on the synthesis of these vitamins (359).

Shetland ponies fed rations composed of beet pulp, corn, purified casein, salt, and vitamins A and D failed to make satisfactory gains except when the ration was supplemented with 10 per cent of yeast, rice straw, or with synthetic riboflavin and pantothenic acid. An intake of 44 mg. of riboflavin per kg. per day was adequate for the normal growth of the ponies under the experimental conditions (360).

*Miscellaneous.*—The total work of frog gastrocnemius muscle has been increased by means of thiamine, nicotinamide, calcium pantothenate, and pyridoxine, but in the case of the last two vitamins the increase was not statistically significant. Work output during the final stages of fatigue, however, was significantly increased by all of these vitamins. Riboflavin neither increased the total work output or the output during the final stages of fatigue (361). Evidence has been obtained that the addition of *dl*-alanine to the basal medium when assaying for various B vitamins with *Lactobacillus casei* somewhat improves the response to the vitamins when present in amounts limiting growth. The effect was especially pronounced in the cases of pyridoxine and folic acid (362). The thiamine, riboflavin, pantothenic acid, and nicotinic acid content of bull semen has been found to be 0.89, 2.09, 3.71, and 3.63 mg. per cc. respectively. The spermatozoa counts were correlated with the concentration of all four vitamins, but initial sperm motility was correlated with the concentrations of thiamine, riboflavin, and nicotinic acid only (363). The average chromosome volume in normal rat organs has been found to closely parallel the total concentration of B vitamins, with the exception of inositol (364).

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## FAT-SOLUBLE VITAMINS

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### GENERAL

The controversy over the merits of generous vitamin feeding rages unabated. Spies (1) has pointed out widespread nutritional deficiency, and has recommended, among other corrective measures, that all margarine be fortified with vitamin A. Takenouti (2) has pointed out that generous levels of all vitamins protect against infection. On the other hand, the experimental feeding of vitamin supplements to normal subjects did not produce any measurable benefits (3, 4, 5). The American Medical Association has opposed indiscriminate use of vitamin concentrates, and their Council on Pharmacy and Chemistry (6) has published a list of comparative costs of commercial vitamin mixtures.

Hamilton & Hogan (7) have reported on the hamster's need for vitamins A, D, E, and K.

### VITAMIN A

*Chemical studies.*—Deuel and co-workers (8) have shown that neo- $\beta$ -carotene has a biological provitamin A activity equivalent to about 38 per cent of that of natural  $\beta$ -carotene. Since the neo- $\beta$ -carotene U studied contained one double bond in *cis* configuration, the authors suggest that only those molecules which are rearranged to the usual all-*trans*- $\beta$ -carotene can be activated in the body. Further work on the *cis-trans* isomerization applied to  $\alpha$ -carotene isomers was reported by Nash & Zscheile (9). Isomerization was produced by the application of heat or by the iodine-light method. Optical properties of the various isomers were recorded. It has also been shown (10) that isomerization of  $\beta$ -carotene increases its optical density at 326 m $\mu$ . This fact must be taken into consideration when determining the correction values to be used for vitamin A analyses by spectrophotometric methods.

It is generally recognized that the usual crude carotene determination may not give a true measure of the provitamin A activity of a sample. Kemmerer and co-workers (11, 12, 13) have reported the



composition of the crude carotene of some forages. Typical data from their report are summarized in Table I.

TABLE I  
CONSTITUENTS OF THE CRUDE CAROTENE OF SOME FORAGES

Material	$\beta$ -Carotene*	Neo- $\beta$ -Carotene U*	Neo- $\beta$ -Carotene B*	Impurity A*	$\beta$ -Carotene Equivalent*
Fresh grasses .....	72.7	12.1	8.8	6.4	77.1
Dormant grasses .....	51.1	15.6	7.8	25.5	55.8
Silages .....	28.9	14.2	5.9	51.0	31.8

\* The values represent percentages of the total crude carotene content.

It has been demonstrated that the carotenols, luteol, and zeaxanthol do not possess vitamin A potency for growing chicks (14).

Popper has used fluorescence microscopy to study the distribution of vitamin A in animal tissues (15). This application of the fluorescence measurement (16) opens a new approach to studies on the distribution of vitamin A and on factors influencing concentration and distribution. Vitamin A fluorescence was not demonstrable in the epithelium where the first morphological signs of vitamin A deficiency appear. Even in extreme deficiency, vitamin A did not completely disappear from the retina.

The difference in intensity of fluorescence under ultraviolet light has been used as a means to analyze mixtures of free vitamin A and vitamin A esters (17). The method is not applicable if carotenoids are present, because these compounds reduce transmission of ultraviolet light and also display fluorescence of their own. Suitable means of separating these carotenoid pigments have not been worked out. By this fluorescence technic various fish liver oils were found to contain 49 to 63.5 per cent of esterified vitamin A. These results have been challenged (18) because other methods indicated that about 95 per cent of the vitamin A in fish liver oils and their distilled concentrates was present in the esterified form. Solvent fractionation and chromatographic methods for the quantitative separation of alcohol and ester forms of vitamin A have been described, and their limitations noted (19). The alcohol and ester forms are apparently about equal in biological activity (20).

Photochemical destruction of carotene occurs in the presence of chlorophyll, methylene blue, eosin, and uranyl acetate (21). The details of the reaction have not been determined, but a carotene peroxide is postulated as an intermediate product of the photolysis.

The preparation of vitamin A aldehyde by oxidation of vitamin A alcohol with aluminum propoxide has been described (22). Further work has been reported on the "cyclization" of vitamin A and allied compounds (23), and the name axerophthene has been suggested for the dehydration product formed by the action of alcoholic hydrochloric acid on vitamin A alcohol (24). Attempts to interconvert  $\alpha$ - and  $\beta$ -carotene by heating with sodium isopropoxide proved unsuccessful (25).

*Assay methods.*—Assay methods for vitamin A and carotene received considerable attention during the year. This attention is well considered since much of the published work has been handicapped by the use of analytical methods which were not sufficiently accurate when applied to food products. Criticism of the International Standard and of the U.S.P. Reference Cod Liver Oil No. 2 have prompted many investigators to use crystalline vitamin A as a reference standard. A report from The Netherlands claims that the International Standard has lost 21 per cent of its potency since 1935 (26).

The reaction with antimony trichloride has been used as the basis for several improved methods of assay. Usually the original Carr-Price technic gives results in good agreement with those obtained by spectrophotometric methods when applied to fish oils. However, interfering substances—which either inhibit color development or themselves react to produce a blue color—sometimes prevent good agreement (27, 28). The use of the unsaponifiable fraction and the plotting of results in terms of extinction ratios ( $E_{\lambda}/E_{328\text{ m}\mu}$ ) rather than extinction coefficients ( $E_{1\%}^{1\text{cm}}$  at 328 m $\mu$ ) has served to make the spectrophotometric method more dependable for use with low potency fish oils. Where the ultraviolet absorption curves of the unsaponifiable extracts of food materials are not typical of vitamin A, Oser, Melnick & Pader (29) recommend the use of an antimony trichloride technic modified to correct for color inhibitors, color and turbidity of extract, etc. Good agreement with the U.S.P. bioassay is claimed when this method is applied to foods and various pharmaceutical preparations (30).

The Carr-Price reaction has been much used for work with blood plasma and similar materials containing both carotene and vitamin A (31, 32). A simple method for the separation of carotene from vitamin A is based upon the differential solubilities of the two in ethyl alcohol. The carotene is precipitated from absolute ethyl alcohol solution by dilution, and separated from the vitamin A by filtration. The

separation is sufficiently complete for the subsequent determination of vitamin A in the filtrate.

While the antimony trichloride method solves some problems, it still leaves others for future work. The reagent must be kept strictly anhydrous and accurately measured volumes must be dispensed quickly because of the unstable color produced. This problem is solved by the use of suitable dispensers such as those described by Oser and co-workers (29) and by Swain (33). While the spectrophotometric values for many low potency vitamin A samples tend to be higher than those obtained by the U.S.P. bioassay technic, there is more than a suspicion that many colorimetric values tend to be too low. Benham (34) suggests that low values are more likely to be due to faulty technic in extraction, washing, and drying, than to decomposition of the vitamin A.

The destructive irradiation technic to obtain a reference base for comparison of ultraviolet absorption values was studied in some detail (35), and applied to determination of the vitamin A content of margarine (36). Wilkie & DeWitt (37) compared the colorimetric and the spectrophotometric methods for the assay of vitamin A in margarine. To purify the extracts, they employed chromatographic adsorption, using a column of celite and magnesium oxide. Sodium hydrosulfite layers were placed at the top and bottom of the column to guard against oxidation, and all operations were carried out in the presence of a reducing agent. Passage of the vitamin A through the column was followed by observing fluorescence under ultraviolet light. These workers found that the spectrophotometric values, using the destructive irradiation technic, tended to be higher than those obtained by the U.S.P. bioassay while the colorimetric assay results tended to be lower.

Two modified bioassay procedures have come to the reviewer's attention. In one of these methods (38) the determination is based on the quantity of vitamin A stored in the livers of rats previously depleted of this vitamin, when the test material is fed on two successive days. On the fourth day the rats were killed and vitamin A was determined in their livers by the Carr-Price method. The method was found to be as accurate as the curative growth test. The other method (39) is based on changes caused by vitamin A depletion in the cellular contents of the vagina. Refinements in technic are credited with improved accuracy over that obtained in previous attempts to utilize this phenomenon for assay purposes. The rats are ovariectomized,

and when squamous cells predominate in the vaginal smears the rats are considered depleted. The doses of standard and sample are fed for two successive days, and the response is taken as the number of days required for the cellular contents of the vagina to change to leucocytes and return to predominately squamous cells (depleted state).

The possible use of certain clays which give a blue color with vitamin A as test reagents for the vitamin has been suggested (40).

Schrenk and co-workers (41) presented a method for the determination of vitamin A in dehydrated eggs, using a spectrophotometric technic with suitable corrections for absorption of ultraviolet irradiation by the carotenoid pigments present.

Refinements in the technic for the determination of carotene have been suggested to reduce losses during assay and to simplify the technic (42 to 45). In reports by Mann (46) and by Kemmerer (47) methods are outlined for the separation and estimation of the various components of the crude carotene determined by usual methods.

The problem of chemical determination of total vitamin A activity in milk and similar products containing both preformed vitamin A and carotene is necessarily complicated. The simplest solution is the use of the bioassay technic, but this is not always practical because of limitations of time or materials. A rapid method for the extraction and determination of vitamin A and carotene in milk has been described (48). Two volumes of milk are mixed with three volumes of alcoholic potassium hydroxide and allowed to stand three hours at room temperature. The mixture is then extracted twice with ethyl ether. Carotene is determined by light absorption at 440 m $\mu$  in petroleum ether. The solvent is evaporated from the colorimeter cell. The residue is taken up in chloroform and antimony trichloride reagent added for determination of the vitamin A. Good agreement with results obtained by longer methods is claimed. A critical study of methods for the determination of vitamin A and carotenoids in butterfat has been reported by Zscheile and co-workers (49, 50). Interference of azo dyes was studied. These can be removed in the carotene determination by extraction with aqueous methanol or diacetone alcohol, but this technic is not applicable in the vitamin A determination. Since the dyes do not seriously interfere, the antimony trichloride reaction is the preferred method available for butter containing such dyes. Correlation with bioassay results was not as good as desired. The authors conclude that more extensive purification of the vitamin

A fraction is needed, especially for application of direct spectrophotometry.

*Stability.*—Bailey (51) discussed the stability of vitamin A during household storage of medicinal oils. He pointed out that oil in capsules is much more stable than in bottles, and that the latter needs to be protected against light and oxidation. Silker and co-workers (52) recommended various treatments to stabilize the carotene in alfalfa during the drying process. They urged preliminary blanching and found that the addition of antioxidants or of chemicals to inactivate enzymes was also helpful. Diphenylamine and hydroquinone were the most effective antioxidants tested, while thiourea and sodium cyanide were the most effective enzyme inactivators. The value of low temperature storage was stressed. The value of preliminary scalding prior to dehydration of vegetables was also pointed out (53). Bickoff & Williams (54) found it necessary to protect carotene in oil added to solid carriers. They found diphenylamine superior to hydroquinone as an antioxidant. When mineral oil was used as a carrier, the stability of the carotene was usually better than when vegetable oils were so used.

It has been reported that the material from which the can was made had no effect upon the stability of carotene in canned foods (55). Milk fat could be stored for several months even at 60° C. without loss of vitamin A or carotene provided the fat had been degassed and tightly sealed in completely filled light-proof containers. Laquered tin cans were no better than plain cans. The study suggests that a relationship exists between the ability of the fat to resist oxidation and the stability of the vitamin A activity (56). When butter oil was oxidized under controlled conditions (57), carotenoid destruction proceeded rapidly during the initial stages of peroxide formation. Some materials treated to remove fatty acid peroxides still retained a marked ability to destroy carotene (58), and vitamin A and carotene were destroyed before rancidity, as measured by peroxide formation, became apparent (59). The destruction was attributed largely to surface oxidation.

Lovern (60) did not consider any antioxidant satisfactory for use with dried foods containing vitamin A and carotene, although a number tested were very helpful when applied to solutions. A patent was obtained (61) for stabilizing vitamins through the use of seed meal extracts as the carrier for the vitamin bearing oil. Taub & Simone (62) found that a mixture of inhibitors such as lecithin,  $\alpha$ -

tocopherol, ascorbic acid, and niacin exert an interlinking antioxidant action. Further work on the antioxidant activity of the tocopherols and the relation to vitamin A will be discussed later in this review under vitamin E.

A study of the effect of spray drying and subsequent storage of eggs by Denton *et al.* has just appeared (63). These workers found no loss during the drying process. Vitamin A was lost rapidly from the stored product, while the potencies of vitamin D and riboflavin were not lowered by storage.

*Symptoms of vitamin A deficiency.*—Extensive use has been made of dark adaptation measurements in attempts to diagnose human vitamin A deficiency. Isaacs and co-workers (64) concluded that the Hecht adaptometer was more reliable for the determination of dark adaptation thresholds than the biophotometer. Dark adaptation tests are useful to detect the general vitamin A nutritional level (65), but most workers urge caution in interpretation of tests (66 to 69). In veterinary practice, the use of vaginal smears for the diagnosis of vitamin A deficiency is recommended (70).

In many attempts to produce experimental human deficiency the depletion is not continued long enough to bring out the usual symptoms of vitamin A deficiency. It has been pointed out that the time required to change nutritional status is related to the body storage of the factor in question (71). Getz (72) found the symptoms of human vitamin A deficiency, in the order of their appearance, to be as follows:

(a) Conjunctival changes which occurred in thirteen weeks on the low vitamin A diet. This symptom was practically cleared in about seven months on a normal diet. (b) Night blindness which developed in twenty-four to twenty-eight weeks of vitamin A fasting. Normal vision was not completely restored in eleven months on a normal diet. (c) Skin changes which occurred after forty-six weeks. The skin changes were reversible and cleared within one month on a normal diet. (d) Lowered plasma vitamin A.

Using dark adaptation measurements, Batchelder & Ebbs (73) found a daily intake of approximately 5000 International Units (I.U.) of vitamin A daily (74 to 84 I.U. per kg.) just sufficient for maintenance near the normal threshold. Sevringhaus (74) also placed the adult requirements at 5000 units per day. Requirements of 25 to 40 I.U. per kg. were suggested by one group of investigators (75, 76), but most workers prefer to recommend vitamin A intake above rather than below the generally accepted standard of 5000 units per day

for normal adults. Increased needs for vitamin A during pregnancy were stressed by Lund & Kimble (77), who recommend in addition to a good diet 5000 I.U. daily during the second trimester and 10,000 during the third trimester. Evidence has been presented to show that the minimum requirements are scarcely being met by persons in the low income groups (78) and that Europeans under war time food restrictions are receiving far less than their requirements (79).

*Bovine requirements.*—Numerous investigators have pointed out the large seasonal variation in the vitamin A content of milk and milk products, and the influence of feed upon this variation (80 to 88). Wide variation in the vitamin A content of beef fat was also shown to be caused by variation in carotene intake (89). In one study (90) the numerical values expressed as mg. of carotene per 100 cc. were nearly the same for blood plasma and for milk fat produced at that time. Such a direct relationship, of course, would not be generally true under varied conditions. Hilton and co-workers (91) placed the requirements for preformed vitamin A at 200,000 I.U. daily when the source was fish liver oil.

While workers are agreed that the vitamin A content of milk can be increased by vitamin A feeding, there is disagreement regarding the effect of vitamin A feeding upon the quantity of milk produced. Fountaine & Bolin (92) found vitamin A feeding to have no effect upon milk or butterfat production. This is in contrast to the results of extensive work summarized by Wilson (93). Under some conditions the addition of fat causes an increase in milk production (88). The finely divided globules of fat which remain with the skim milk and with the whey contain seven and eleven times, respectively, as much carotenoids as the original milk fat (94).

Moore & Berry (95) found the colostrum essential to raise the plasma vitamin A and carotene in calves. When colostrum was withheld and whole milk substituted, the calves' plasma levels showed little increase and most of the animals died of infection. The blood plasma vitamin A levels were quite low at birth (2.4 to 4.2  $\mu$ g. vitamin A and 1.5 to 3.4  $\mu$ g. carotene per 100 ml.) but showed a fivefold increase with the intake of colostrum during the first twenty-four hours and reached maximal levels at about three days of age.

Improved health and slightly better growth was observed following the addition of cod-liver oil to the rations fed to calves to six months of age (96). For feeder cattle it was reported (97) that 450 mg. of carotene per 100 lbs. live weight was not sufficient to maintain life,

that a daily intake of 1500 mg. per 100 lbs. was the minimum level, and that a daily intake of 2000 to 2500 mg. per 100 lbs. was recommended. Lewis & Wilson (98) found that a daily intake of 64 units of preformed vitamin A per kg. body weight was the minimum that would permit maximum growth of dairy calves. If this were considered the requirement for growth of market beef it would be equivalent to about 29,000 units per 1000 lbs. live weight. For maximum blood vitamin A levels these workers found it necessary to feed 512 units per kg., and for substantial liver storage 1024 units per kg. per day.

*Utilization of carotene.*—Some of the complications arising from the use of carotene as a reference standard for vitamin A have already been reviewed (16). Aside from possible effects due to vitamin E, there seems to be a species difference in the utilization of carotene. While the rat, at least with ample vitamin E intake (99), is generally supposed to be an efficient converter of carotene into vitamin A, one has only to check the feces to note that 10 to 15 per cent of the  $\beta$ -carotene administered in small doses is excreted unchanged (100). Shaw & Deuel (101) observed that the rate of absorption of carotene from the rat intestine is proportional to the dose fed, and as rapid as the absorption of vitamin A *per se*.

Man does not absorb carotene efficiently (102) and this should cast doubt on the suggestion that alfalfa be prepared as a vegetable (103). Getz (72) described experimental work on humans in which he used a diet low in vitamin A but high in carotene. The carotene content of the blood rose to 900 to 1100  $\mu$ g. per 100 ml. of plasma, but the vitamin A levels did not rise above 170 I.U. per 100 ml. These were the maximum levels reached with an intake of 200,000 I.U. of carotene per day. The data indicated relatively poor conversion of carotene to vitamin A. Getz estimated this conversion at 15 to 20 per cent. Carotene is efficiently utilized by the chick (104), rabbit (105), and lamb (106). The horse is reported to be inefficient in converting carotene to vitamin A, and the normal plasma vitamin A level is only about  $12.5 \pm 3.5$   $\mu$ g. per 100 ml. (107). For the dairy cow, vitamin A from fish liver oil was found to be nearly three times as effective as carotene in dehydrated alfalfa (91), and no differences were found in the utilization of carotene in oil solution or from alfalfa (108).

*Acetonemia.*—Acetonemia in dairy cattle has been successfully treated with vitamin A (109, 110, 111). Chemical studies indicated that cows with acetonemia had very low blood vitamin A levels (around 4. I.U. per 100 cc. of plasma) and exceptionally high blood



carotene levels (average, 1035 I.U. per 100 cc.) (112). These observations suggest that acetonemia may involve poor conversion of carotene to vitamin A. Administration of large doses of vitamin A by mouth produced marked improvement in the cow's condition, accompanied by increased blood vitamin A and decreased blood carotene levels. This is in line with previous reports which have shown that high vitamin A intake tends to lower the carotene levels (87, 92). It must be kept in mind that some of these cows (110, 112) were kept under conditions where their intake of vitamin A was exceptionally low, and further work is required to show clearly that vitamin A deficiency *per se*, or loss of ability to convert carotene to vitamin A due possibly to deranged liver function, is the primary cause of all cases of acetonemia. Human blood vitamin A is low in cases of liver damage (113, 114, 115).

*Functions in health and disease.*—Steigmann & Popper (116) found that the shape of the tolerance curve is similar for vitamin A alcohol and esters, while ingestion of carotene has little effect upon the plasma A levels. The shape of the tolerance curve is not necessarily related to the fasting plasma vitamin A level and is not influenced by administration of vitamin E. Some cases of abnormal blood protein pictures are helped by administration of vitamin A (117). Thrombocyte count is not influenced by vitamin A, although vegetable oils used as carriers in some vitamin A preparations do cause an increase in thrombocytes (118). Carotene appears to activate insulin and may participate in cellular oxidation processes (119). It is suggested that carotene solution should be administered to supplement insulin therapy in diabetes. Vitamin A is concerned in glandular function (120 to 123), but the exact nature of the role which vitamin A plays is not known. Prolonged deficiency of vitamin A increases susceptibility to parasites (124, 125), and to dysentery (126, 127). Vitamin A is not a detoxifying agent, but deficiency lowers resistance to toxic agents (128).

With diets suboptimal in vitamin A, the addition of 40 mg. per cent of atabrine did not cause a further reduction in the growth rate of experimental rats (129). This is in contrast with studies on riboflavin and protein. When vitamin A was injected with solutions of dibenzanthracene the toxic action of the latter was reduced (130). Vitamin A administration had little effect upon renal function (131), except that massive doses did produce a slight increase in filtration rate. Katz and co-workers (132) found vitamin A did not influence hypertension

in the dog. This is in line with work previously reviewed (133), and in contrast to the favorable observations of Villaverde (134).

Getz and co-workers (135) noted that patients suffering from tuberculosis had normal blood carotene levels and abnormally low blood vitamin A levels. They postulate that patients with tuberculosis may need additional amounts of vitamin A. A review dealing with the administration of massive doses of vitamin A in tuberculous diabetes has recently appeared (136).

It has been shown that nutrition is a conditioning factor predisposing to rheumatic fever (137), and that at the onset of the disease there is a fall in the level of plasma vitamin A (138). The plasma carotene level was not significantly altered. Whether the disturbed vitamin A metabolism is a causative factor or merely a result of the process remains for further investigations to determine (139).

In a study on the relation of vitamin A to color vision (140), dubious improvement occurred in one out of thirteen persons tested. Vitamin A treatment relieved eye strain in a small percentage of the cases of presumed deficiency (141).

*Nutrition of the fetus.*—Both carotene and vitamin A pass through the placenta, but only in limited amounts (142). Evidence has also been presented to show that the levels of these in fetal blood cannot be raised appreciably by feeding vitamin A to the mother (143, 144). A report of normal blood vitamin A levels at birth (145) raised the question whether the cord blood should be considered as representative of the newborn. Lower levels were found during the following three days. Warkany & Schraffenberger (146) reported congenital malformations of the eyes induced in rats by maternal vitamin A deficiency. A review has recently appeared which discussed rather fully the dietary causes of congenital abnormalities and clinical studies on prenatal nutrition (147).

*Liver storage and depletion.*—The long time required for depletion of human body stores of vitamin A has already been stressed. Other studies have shown that 1200 I.U. stored in a rat's liver can be depleted in twenty-four weeks on a vitamin A deficient diet (148), and that some storage can occur in previously depleted rats when they are fed doses of 8.4 I.U. daily (149). Clayton & Baumann (150) found that hepatic storage of vitamin A was comparatively independent of other processes taking place in the liver. Among the factors found not to influence the rate of storage or depletion of vitamin A might be noted the following: carcinogenic agents, vitamin K, coumarin deriva-

tives, accumulation and flushing out of fat deposits, and choline deficiency. Some of these findings contrast with others reported elsewhere in this review.

*Hypervitaminosis.*—It has been shown that excess vitamin A has no effect on dark adaptation (151). While there is little danger of overdosage of vitamin A under normal conditions, a number of reports do point out the dangerous possibilities. Rodahl & Moore (152) attribute the toxicity of bear and seal livers to their high vitamin A content. Ingestion of large quantities of high potency fish liver or liver oils would likewise be dangerous. Telang livers were found by Herbst and co-workers (153) to have a higher vitamin A content than normal beef liver. When this was fed to young rats at levels supplying more than 15,000 I.U. per day it proved to be toxic. Identical symptoms were produced by feeding similar levels of crystalline vitamin A. Light and co-workers (154) noted that overdosage of vitamin A results in a hypoprothrombinemia, which can be corrected by daily administration of vitamin K. This treatment, of course, has no effect upon other symptoms due to hypervitaminosis A. Josephs (155) in a review of the literature on hypervitaminosis A and carotenemia points out that large intakes of carotene are comparatively harmless. However, high carotene intake can cause intense skin pigmentation (156).

*Vitamin A and farm animals.*—Vitamin A deficiency has been blamed for development of paresis (120) and cardiac failure in swine (157). Studies of feeding methods to meet the requirements of growing pigs (158) and to raise the content of vitamin A in colostrum (159) have been reported. Ellis (160) points out that if the breeding stock has ample vitamin A, pigs weaned from such sows will not usually show vitamin A deficiency during the growth and fattening period. A similar protection is afforded in the case of beef cattle. Such deficiencies of vitamin A as may develop do not seriously affect the efficiency of feed utilization. An apparently greater need was pointed out in the earlier work of Barron (161).

The importance of vitamin A for poultry was accentuated by the recent shortage of this factor. Allowances recommended by the National Research Council (162) are expressed as follows in terms of International Units of vitamin A activity per pound of feed: starting chicks, 1200; laying and breeding hens, 3300; poults, 2500; turkey breeders, 4000.

*Distribution and concentration.*—Reports have appeared on the distribution of carotene in various plants and feedstuffs (163 to 166),

in corn distillers' by-products (167), and in *Rhodotorula* (168). Pollen was reported to have considerable vitamin A activity (169, 170). Data on the vitamin A activity of canned foods were accumulated by Pressley and co-workers (171). Preparations of carotene rich foods from aquatic plants has been suggested as a means to combat vitamin A deficiency in India (172). A method for industrial preparation of carotene concentrates from vegetable leaf wastes, with recovery of 85 to 95 per cent of the carotene, has been described by Wall *et al.* (173). The methods, in general, follow principles in use for assay of carotene, with modifications to adapt them for large scale operation.

Porpoise livers were found to contain both carotene and vitamin A in substantial but variable quantities (174). Data on vitamin A and D contents of South African fish products were accumulated by Rapson *et al.* (175, 176). Springer & French (177) found liver oil samples from sharks and rays of the Florida region to vary in vitamin A potency from 35 to 340,000 U.S.P. units of vitamin A per gm. Their results are tabulated according to species. The use of xylene as a solvent for extraction of oil and vitamin A in routine examination of shark livers was recommended by Sycheff (178). It was not necessary to remove the xylene prior to testing by the Rosenthal-Weltner reaction (179). The color developed by the latter is reported to be stable for twenty to thirty minutes at room temperature, and this represents quite an advantage over the unstable colors developed by the usual antimony trichloride method.

#### VITAMIN D

*Assay.*—Various attempts have been made to replace the biological assay methods with chemical procedures. Shantz (180) studied the antimony trichloride reaction with vitamin D, using crystalline calciferol as the test material. To obtain reproducible results he found that the conditions of concentration, time, light, and temperature must be rigidly controlled. Peterson & Harvey (181) used the color development when concentrated sulfuric acid was added to a carbon tetrachloride solution of ergosterol to measure the concentration of this provitamin. Absorption spectra (182) do not offer much hope for an assay method applicable to food products. Beall & Grant (183) described a color reaction with ferric chloride in sulfuric acid. The vitamin D was added in chloroform solution. A green color was produced. The method offered some promise of a means to distinguish between vitamins D<sub>2</sub> and D<sub>3</sub> because there was less color produced with vitamin

D<sub>2</sub>. However, the report did not clearly indicate how to distinguish between the lesser color due to vitamin D<sub>2</sub> and concentration of vitamin D<sub>3</sub>. A color reaction is given by vitamins D<sub>2</sub> and D<sub>3</sub> on addition of glycerol dichlorohydrin or related compounds in the presence of acetyl chloride or other halides of acid nature (184). Distinguishable color reactions were given by ergosterol and by 7-dehydrocholesterol, but apparently identical reactions are given by vitamins D<sub>2</sub> and D<sub>3</sub>. While these and other chemical methods are applicable to nearly pure sterols, there is as yet no substitute for the use of biological assays to measure the vitamin D activity of food products or even of usual high-potency concentrates of the vitamin. A number of attempts have been made to improve and simplify the bioassay methods, but none that have come to the attention of the reviewer can be considered to represent fundamental changes in either the principles or the results obtainable.

Jones & Elliot (185) advocated the use of growth response of chicks as the criterion for assay of vitamin D<sub>3</sub>. This requires care in selection of the rachitogenic diet and standardization of conditions, but may not require as much mathematical calculation to evaluate the results. Motzok & Hill (186) studied factors influencing the chick vitamin D assay. They concluded that freezing the bones lowered ash content while storage in 95 per cent ethyl alcohol was without effect, that crushing the bones prior to solvent extraction had no effect upon the results, that immersion in boiling water for more than one minute lowered the bone ash, and that ashing for one hour at 850° C. was adequate. Some of these conclusions are at variance with others that have been reported (187). If any cleaning is done prior to immersion of the leg section in boiling water, the entire purpose of the cooking is lost, and this step might well be eliminated. Its use does, however, speed up the operation to a marked extent, and properly applied does not interfere with the accuracy of the method.

Evans & St. John (188) found that good results could be obtained by ashing the toes of assay chicks, either with or without prior solvent extraction. They found the toes slightly more sensitive than the tibiae. Comparable results were obtained by using the per cent ash in the shaft, distal cartilage, extracted toe, unextracted toe, or tibiae. The study was extended to turkey poults with good results (189).

Work has been done on the use of crystalline vitamin D<sub>3</sub> as a standard for the chick vitamin D assay (190), and Kennedy (191) has reported the following activity of the pure vitamin D<sub>3</sub> when assayed against U.S.P. reference cod-liver oil No. 2:

Collaborators' evaluation .....	47,541 A.O.A.C. units per milligram
Free hand response curve.....	50,828 A.O.A.C. units per milligram
Log dose response curve.....	47,262 A.O.A.C. units per milligram
By least squares method.....	51,255 A.O.A.C. units per milligram
Grand average, collaborative data....	49,222 A.O.A.C. units per milligram
Separate DuPont data .....	48,939 A.O.A.C. units per milligram

More uniform response to the vitamin D<sub>3</sub> was claimed.

Oser (192) has commented on the variations within the chick vitamin D assay. Within any one week, using chicks from one uniform source, different laboratories found as much as 7 per cent difference in the bone ash content of chicks in the negative control groups. The increment in bone ash due to the addition of 10 A.O.A.C. units of vitamin D per 100 gm. of diet ranged from 2 to 3 per cent up to as much as 7 to 9 per cent. The variation between laboratories was much greater than between lots of chicks within any one laboratory. Oser suggested that attention be focused upon conditions within the laboratories, such as: variations in diet composition, size of cages, feeder space, temperature, illumination, ventilation, training of chicks to eat, dose range used, and interpretation of the data.

Willgeroth and co-workers (193) suggested the use of turkey poults for the assay of vitamin D because the bone ash range was approximately twice as great as that of chicks and the variations within groups were no greater than with chicks. With no vitamin D the per cent of ash in the dry, fat extracted tibiae was 23.97 to 26.77 and an increase of about 20 per cent ash was obtained with 60 to 75 A.O.A.C. units of vitamin D per 100 gm. of diet. In simultaneous assays, essentially the same results were obtained with poults and with chicks on the various samples tested.

Wallis (194) suggested that low potency samples be incorporated in the basal diet for assay by the U.S.P. rat curative method, and that the mineral intake be appropriately adjusted so that the intake of minerals is the same in the regular rachitogenic and in the supplemented diet as consumed by the rats. Using this technic for testing cows, Wallis found that the vitamin D potency per gram of butterfat decreased as lactation progressed but that the percentage of butterfat increased so that the vitamin D content per quart of milk remained quite uniform for each cow studied. Only about 0.5 to 1.5 per cent of the ingested vitamin D was recovered in the milk.

*Stability.*—The relatively unstable nature of vitamin D is now generally recognized. Huber & Barlow (195) have contributed studies

on the crystalline vitamins  $D_2$  and  $D_3$ , and they suggest that the esters are more stable than the free vitamins. Reports by Milby & Thompson (196, 197) indicate that care must be exercised in mixing D-activated animal sterols with poultry feed ingredients. Fortunately most of the common ingredients were not found especially detrimental to the added vitamin D, but contact with minerals should be avoided as much as possible. Premixing with minerals causes rapid loss of the vitamin D.

*Relation to other factors.*—Sulphur added to poultry diets at levels of 2.5 to 5 per cent raised the birds' vitamin D requirements from 50 to between 175 and 200 A.O.A.C. units per 100 gm. of diet (198). Sunlight or ample dietary vitamin D will prevent "sulphur rickets" (199). Magnesium salts were reported to have a beneficial effect on experimental rickets in rats (200).

The addition of 20 per cent yeast to the diet caused rickets in pigs (201). The rickets could be completely prevented by addition of vitamin D, or partially prevented by addition of calcium. The authors suggest that the rachitogenic effect of yeast is not entirely explained by its high content of available phosphorus. The addition of choline to the basal diet improves the uniformity of healing of rickets in the rat (202). Slightly improved utilization of vitamin D is claimed. This is in line with a reported need for choline to insure normal vitamin A metabolism (203).

*Physiological effects.*—Lambs are born with enough vitamin D to protect them against rickets for about six weeks (204), and rickets do not develop unless there is a moderate degree of growth together with serum calcium levels below 7 mg. per 100 ml. Vitamin D may act to stimulate the formation of an active compound of phosphorus, named phosphagen, and distinct from the true inorganic phosphorus which is reported to remain relatively constant during development and cure of rickets (205).

Administration of vitamin D to rats on a diet low in phosphorus and high in calcium resulted in pronounced hypercalcemia. The data are interpreted (206) to favor the view that vitamin D acts to increase absorption of calcium from the intestines and to emphasize the importance of the calcium-phosphorus concentration product of the serum in calcification. Vitamins  $D_2$  and  $D_3$  were effective in dosage as low as 1 I.U. per gm. of diet, and the dihydrotachysterol was much less effective. Mellanby (207) suggests that vitamins A and D work together in bone growth: vitamin A controls the activity of the osteo-

blasts which lay down the soft bone; vitamin D then governs the deposition of calcium phosphate to harden the bone. Maternal vitamin D deficiency (208, 209) caused congenital skeletal malformations in 45 per cent of the young. Bones were curved and shortened, and sometimes ribs were thickened. The symptoms were not typical of rickets.

The incidence of keel bone deformity in young chickens varied inversely with the level of vitamin D supplied (210). Removal of the green gland of chickens had no effect upon the development of rickets on a diet lacking vitamin D (211).

After administration of vitamin D, the blood sugar curve reaches its maximum more quickly following ingestion of glucose and also returns to normal more rapidly (212). This is attributed to accelerated absorption from the intestines. Irving (213) did not consider the action of vitamin D to be restricted to the effect upon intestinal absorption. He found that the calcification of the teeth was dependent upon vitamin D, and that the teeth were more sensitive and responded more quickly to vitamin D than did the epiphyses. On the other hand, Day (214, 215) found a very low incidence of dental caries in children with rickets and women with osteomalacia, which led him to conclude that vitamin D deficiency was not a factor in the etiology of dental caries. Previous work (216) has shown vitamin D to be a factor in tooth formation in the rat. However, the work on animals has not been related to that on humans (16). Large doses of vitamin D were not detrimental.

While large doses of vitamin D were more toxic to adrenalectomized rats (217), there was no evidence that such doses increased the activity of the thyroids. Toxicity was noted in doses as small as 300 I.U. administered daily to growing rats, and females seemed to be more susceptible than males. McChesney (218) has studied further the toxicity of various activated sterols using mature rats. He found the following dosage, expressed as milligrams per kilogram per day, permitted a median twenty-day survival period: 3.60 of vitamin D<sub>2</sub>, 2.30 of vitamin D<sub>3</sub>, and 1.00 of dihydrotachysterol. Heywang (219) found that hens receiving generous dietary vitamin D produced eggs with significantly poorer hatchability when they were also exposed to direct sunlight. It is not entirely clear whether this can be considered due to excess vitamin D, or possibly to other factors not well understood.

McChesney (220) has shown that vitamins D<sub>2</sub> and D<sub>3</sub> are more effective in the chick when administered by intramuscular or intra-



venous injection than when given by mouth. The reasons for the greater effectiveness are not explained, but may be related to efficiency of absorption from the digestive tract.

Lecoq (221, 222) believes that mineral imbalance, resulting in alkalosis, rather than a poor ratio of calcium and phosphorus *per se* is the cause of rickets. Although the acid-base balance has received considerable attention (223), few workers consider it of first importance in the etiology of rickets.

Little has appeared recently on human requirements for vitamin D. Clausen (224) has recommended the administration of 800 I.U. daily for prophylaxis—the vitamin preferably in concentrated form. This is within the usual range recommended in this country (225).

*Vitamin D requirements of domestic species.*—Supplying the vitamin D requirements of four-footed animals does not impose a heavy economic burden since all forms of vitamin D are apparently well utilized by such animals (226, 227). For poultry nutrition, however, vitamin D<sub>2</sub> is relatively ineffective and the cost of supplementing rations with vitamin D is considerable. The situation is further aggravated because poultry are less likely to receive sufficient exposure to ultraviolet radiation and because their body stores of vitamin D are usually small.

It has been reported that fish-liver vitamin D is less effective for turkey poult than D-activated animal sterols (228) or irradiated 7-dehydrocholesterol (229, 230). These reports conflict with other data (193), and with practical experience which prompted the National Research Council to recommend allowances as low as 360 A.O.A.C. units per pound of feed for poult without specifying the source of the vitamin D (162). It is difficult to understand these reports because we have considered the vitamin D activity of fish oils, especially when fed to poultry, to be dependent almost entirely upon the vitamin D<sub>3</sub> content of these oils. On this premise it is difficult to see how or why pure vitamin D<sub>3</sub> should be more effective than the same quantity of vitamin D<sub>3</sub> in the form of fish oil. Further work is required to clarify the situation. In the meantime it is apparent that vitamin D requirements of the poult are influenced more than we formerly thought by the mineral content of the ration. It has been shown that minimum levels of phosphorus raise the vitamin D requirements (231), and that not all forms of phosphorus are equally well utilized (232, 233). When minerals are lacking, vitamin D cannot prevent rickets (234).

Table II illustrates the effect of varying the mineral content of the poult's diet when the vitamin D intake is kept constant at 70 A.O.A.C. units from tuna-liver sources. This is a minimum level, according to all reports in the literature, and normal calcification was obtained only with mineral intake above the usual levels (235). There is some evidence, too, that the lesser effectiveness of fish oils is most apparent at the lower mineral intake, although it is difficult to see how mineral balance could influence the relative effectiveness of different sources of vitamin D.

TABLE II

EFFECT ON TURKEY POULTS OF VARYING THE MINERAL CONTENT OF A DIET CONTAINING 70 A.O.A.C. UNITS OF VITAMIN D FROM TUNA LIVERS PER 100 GM. OF DIET

Supplement to Diet	Calcium*	Phosphorus*	Bone ash†	Wt. gain‡
None .....	0.35	0.60	22.08	67
1% CaCO <sub>3</sub> .....	0.74	0.60	24.12	100
2% CaCO <sub>3</sub> .....	1.14	0.59	26.92	99
3% CaCO <sub>3</sub> .....	1.53	0.59	34.73	160
4% CaCO <sub>3</sub> .....	1.92	0.58	38.41	141
2% Bone meal .....	0.93	0.87	28.88	76
2% CaCO <sub>3</sub> + 2% Bone meal ....	1.72	0.87	33.53	100
4% CaCO <sub>3</sub> + 2% Bone meal ....	2.50	0.87	40.27	124
6% CaCO <sub>3</sub> + 2% Bone meal ....	3.28	0.87	43.21	133
3% CaCO <sub>3</sub> + 3% Bone meal ....	2.39	1.00	38.43	110

\* Values represent per cent in diet.

† Values represent percentage in dry, fat-free bones.

‡ Grams gain during three week test period.

*Sources and distribution of vitamin D activity.*—Ziegler & Keevil (236) found that irradiation of milk to enrich it with vitamin D caused a loss of 5 to 8 per cent of the riboflavin. In view of other processing losses this cannot be considered serious. Oppel reported Russian work showing that photochemical transformation of ergosterol into vitamin D<sub>2</sub> is apparently a unimolecular reaction (237), and described efforts to prepare hydrosols of vitamin D for parenteral administration.

Approximately 1 per cent of ergosterol has been found in dry *Penicillium* mycelium of several species (238, 239). This might indicate that the mold could be irradiated as a practical source of vitamin D<sub>2</sub>.

Fluorescent lights were found to emit radiation which promoted calcification in the chick while ordinary inside frosted incandescent bulbs did not promote calcification (240).

## VITAMIN E

*Determination and chemical studies.*—Emerson & Evans (241) have attempted to standardize and improve the accuracy of the bioassay method for vitamin E. Low fat, purified rations were superior to the usual high fat ration, and feeding the supplement over a period of several days provided a more sensitive test than did the single dose technic. When determining tocopherol with ferrous iron salts and  $\alpha$ ,  $\alpha'$ -dipyrrine, a blank can be obtained by acetylation (242) which destroys the activity of the tocopherol and allows the measurement of interfering substances. For analysis of wheat-germ oil and similar sources, removal of interfering material with Frankonit S has been recommended (243). Bleaching earths containing large amounts of aluminum oxide should not be used since these may remove tocopherol. Minot (244) has adapted the dipyridyl method to the determination of tocopherol in blood serum. Tocopherol concentrates may be prepared by high vacuum distillation (245) or by low temperature crystallization technics (246) from suitable tocopherol bearing oils.

*Relation to hormones.*—In castrated animals large doses of vitamin E have a weak progesterone effect (247), which is probably due to an indirect effect on the adrenal cortex. Vitamin E is involved in the functioning of the corpus luteum hormone (248). It appears to stimulate the maturing of the primordial follicles in the ovaries (249). It has also been reported that vitamin E does not act directly on the ovary, but rather by way of stimulating the gonadotropic hormone of the anterior pituitary (250). Ershoff (251) has reported degenerative changes in the corpora lutea of vitamin E deficient rats as early as the sixteenth day of pregnancy.

*Body storage.*—Lundberg and co-workers (252) used the antioxygenic effect of  $\alpha$ -tocopherol as a measure of tocopherol content of abdominal fats. The induction period prior to absorption of oxygen in a standard Warburg apparatus was noted. The amounts of tocopherol found indicate that the fat deposits may at times be major sites of storage. Maximum deposition was not noted until seven to ten days after feeding a single 50 mg. dose.

*Tocopherols as stabilizing agents.*—It is well recognized that the tocopherols can be used as antioxidants for fats, and that vegetable oils (253) or concentrates made from them (254) are effective in preserving animal fats largely because of their tocopherol content.  $\alpha$ -Tocopherol may be used as an antioxidant to preserve vitamin A in fish oils,

but it is doubtful if the antioxidative behavior of crude fish-liver oils is due predominately to  $\alpha$ -tocopherol naturally present (255), and solvent extracts of crude vegetable oils appear to be more effective than their vitamin E content would indicate (256).

The effect of vitamin E on the utilization of other vitamins has been reviewed by Hickman (16), but numerous papers have appeared on the subject during the past two years. When fats low in tocopherol are used in the basal diet for assay of vitamin A, the growth is dependent upon the vitamin E supplied (257), and the utilization of carotene varies according to the vitamin E content of the material tested (99). While vitamin A added to carotene in oil promotes bleaching by aeration, the addition of vitamin E preserves the carotene because the vitamin A acts as a pro-oxidant while vitamin E acts as an antioxidant (71). Simultaneous feeding of vitamin E with the vitamin A to dairy cows is reported to prevent the drop in carotene levels in the blood and milk, by a similar action within the cow. A series of reports from the Distillation Products Laboratory (258, 259, 260) summarize our present knowledge of the sparing action of the tocopherols on vitamin A and carotene. Probably the sparing action is due chiefly to prevention of oxidation in the digestive tract (99, 260), and more carotene and other oxidizable substances can be recovered in the feces following administration of tocopherol simultaneously with the carotene. Alpha-, beta-, and gamma-tocopherols are equally effective, and there is some evidence that a mixture of the tocopherols may be more active than any one alone. The free tocopherols are more effective than their esters.

*Chick experiments.*—Dam (261) has shown that the symptoms of vitamin E deficiency in the chick—exudative diathesis and encephalomalacia—can be developed or suppressed by changing dietary factors other than the vitamin E content of the diet. Purified diets containing only traces of fat rarely produce either symptom. Other species do not show these symptoms. Fatty acids from cod-liver oil, lard, and linseed oil, as well as commercial unsaturated  $C_{20}$  acids added to the diet tend to produce exudates, whereas fatty acids from hog liver tend to produce encephalomalacia. The most unsaturated fraction from hog liver (262) proved to be especially toxic to chicks, 0.5 to 0.6 per cent being effective in producing symptoms and 4 per cent causing a rapid development of encephalomalacia and death. The toxic action was counteracted by administration of  $\alpha$ -tocopherol.

Feeding of  $\alpha$ -tocopherol did not influence the deposition of cholesterol in the aorta in either chicks or rabbits on high cholesterol diets

(263), but did prevent high mortality in the latter species. Dam has also shown (264) that generous levels of vitamin E in the diet of rats increases the survival time of this species on protein deficient rations. The interpretation of these observations is somewhat obscure. It is apparent that vitamin E has a profound influence on general health and body processes, but it is not clear whether this influence is due to control of oxidation within the body or to other functions as well.

Patrick & Morgan (265) observed that on a vitamin E deficient ration the efficiency of feed utilization was influenced to a marked degree by the feeding of *dl*- $\alpha$ -tocopherol. They believe that field encephalomalacia is probably a vitamin A deficiency caused by destruction of the vitamin A and carotene in the feed (59). Patrick (266) criticized the following technics used to produce vitamin E deficiency: (a) ferric chloride may destroy factors other than vitamin E; (b) rancidity may destroy factors other than vitamin E—encephalomalacia may develop on rancidity diets; (c) simplified diets may be deficient in factors other than vitamin E, and on such diets general edema develops. For the production of the crazy chick syndrome, there must be 80 to 100 units of vitamin A in the liver when the chick is hatched. If there is less storage, death occurs without specific symptoms. Patrick & Morgan (267) conclude that vitamin E, *per se*, is not required by the chick during the first eight weeks of life, and that any value of the vitamin E is due to its preservation of vitamin A and carotene. They also point out that addition of vitamin E to a ration may not be desirable. The vitamin E may be oxidized to a quinone which in turn may act as a pro-oxidant for vitamin A.

*Medical applications.*—It has been shown that the addition of heated lard to a ration produces paralytic symptoms (268), but it is not clear whether or not this merely increases the requirement for vitamin E. The normal human serum levels have been given as 0.19 mg. per 100 cc. for men and 0.22 mg. for women (269). Somewhat higher levels were observed by Couperus (270) in studies which indicated that human creatinuria, muscular dystrophy, and amyotrophic sclerosis were not influenced by administration of vitamin E. Minot & Frank (271) found that children with muscular dystrophy had serum tocopherol values of 0.73 to 1.28 mg. per cent, a range fully equal to their normal controls, and that these patients did not respond to vitamin E therapy. Yow (272) has also questioned whether clinical muscular dystrophies can be considered as due to vitamin E deficiency. Somewhat more encouraging results have been obtained by the use of

vitamin E to combat sterility (273, 274) and to prevent abortion (275, 276).

In rat experiments, Aloisi (277) found that muscular dystrophy of the mother leads to abortion. Kaunitz and co-workers (278) noted that administration of 0.5 to 1.0 mg. of *dl*- $\alpha$ -tocopherol to the offspring of vitamin-E-depleted mothers had a protracted effect as measured by the time required before testicular degeneration became apparent. Damage to the rat's reproductive system by vitamin E deficiency was described (279), and induced sterility in the male was not cured by vitamin E feeding (280). The rat's ability to work was improved more by administration of  $\alpha$ -tocopherol than by feeding wheat-germ oil (281). Work increased atrophy of testicles in vitamin E deficiency. In the vitamin E deficient rabbit, the sudden death in advanced muscular dystrophy is due to myocardial failure (282). Such rabbits show greatly increased sensitivity to posterior pituitary extracts. Sweeten (283) described uterine discoloration in rats with chronic vitamin E deficiency. Vitamin E was not curative unless accompanied by successful pregnancy.

Vitamin E has been used with apparent success in treating diphtheria (284). Its antiparalytic and antitoxic effects are the bases for its use.

### VITAMIN K

*New forms.*—Various water-soluble forms of vitamin K have been prepared with various results as regards activity and stability (16). A bisulfite addition product (285) was prepared by heating 2-methyl-1,4-naphthoquinone with a 10 per cent solution of sodium bisulfite for two to three hours at 60°, and the product was claimed to have equal antihemorrhagic activity with less toxicity than 2-methyl-1,4-naphthoquinone. Other work (286) indicates that this derivative is somewhat more toxic than tetrasodium 2-methyl-1,4-naphthaquinol diphosphate hexahydrate which was even more active on an equimolar basis.

Further work has been reported on vitamin K<sub>3</sub>, obtained from the corn stigma (287, 288), but claims for activity superior to that of other known antihemorrhagic factors need confirmation. Vitamin K<sub>3</sub> is claimed to speed up the coagulation of normal blood, but it has been shown that other vitamin K products also render plasma hypercoagulable when administered in doses above 10 mg. per kg. (289).

*Physiological action in the body.*—It has been shown that vitamin K activates trypsin (290). Vitamin K is essential for proper function

of the liver cell in the formation of prothrombin (291). Richert (292) showed that rabbits and chickens were able to convert such antihemorrhagic compounds as 4-amino-2-methyl-1-naphthol, 2-methyl-1,4-naphthohydroquinone diphosphate, and 2-methyl-1-tetralone into 2-methyl-1,4-naphthoquinone. Compounds with labile methyl groups, such as choline, were found to have a slight sparing action on vitamin K (293). Emmel & Dam (294) produced hypoprothrombinemia in chicks without histological evidence of liver damage, either by feeding a vitamin K-free ration or by adding dicumarol to a standard commercial diet. Other dietary deficiencies may explain liver damage reported in earlier work. A relationship between vitamin K, plasma protein (295), and complement function (296) has been suggested. Treatment with vitamin K may produce an abnormal agglutination which should be watched in blood transfusions (297). Shemyakin and co-workers (298) continue to consider the actual vitamin K to be phthalic acid formed from the various active analogs. Karrer & Koller (299), however, were unable to obtain vitamin K activity from the phthalates.

*Medical aspects of vitamin K.*—The medical applications of vitamin K have been reviewed by Dam (300) and by Larsen (301). Dam & Doisy have summarized the uses in medicine to which vitamin K has been put and have indicated what further developments may be expected (302). In rats made hypertensive by wrapping both kidneys with silk, 2-methyl-1,4-naphthoquinone was effective in lowering blood pressure while 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt was not effective (303). Beneficial results followed administration of vitamin K to patients with essential hypertension (304). The prophylactic administration to newborn infants (305, 306, 307) and for older children (308), especially in cases of gastrointestinal disorders (309), has met with widespread approval. Intramuscular injection is preferred to oral administration (310). Prophylactic administration to the mother is recommended (311, 312). A scale for grading hypoprothrombinemia has been proposed (313). For the human adult a dose of 0.01 gm. was effective intramuscularly. Vitamin K prevented hemorrhage in a dog with biliary fistula (314). Vitamin K was not effective in checking diarrhea (315) or hemorrhagic tendencies (316) in patients suffering from tuberculosis. Disturbance of vitamin K equilibrium within the body is reported to cause ulceration of the intestinal mucosa (317).

*Antivitamins.*—Vitamin K appears to be effective against hypoprothrombinemia from any cause, but a number of compounds are able

to counteract its effect. Kornberg and co-workers (318, 319) have studied the production of vitamin K deficiency by administration of sulfonamides, and they conclude that inhibition of intestinal bacterial synthesis of vitamin K is the dominant factor in such production of vitamin K deficiency. Exposure to tropical heat makes animals, and probably humans, more prone to develop vitamin K deficiency and increases the dietary requirements for this vitamin (320). The action of 3,3'-methylenebis (4-hydroxycoumarin) in producing hypoprothrombinemia is accentuated by vitamin C deficiency (321). Derivatives of 3,3'-methylenebis (4-hydroxycoumarin) show much lower hemorrhagic activity than 3,3'-methylenebis (4-hydroxycoumarin) (322) and may even have antihemorrhagic properties (323). The salicylates have been shown to have hemorrhagic activity (289, 324), and 1 mg. of 2-methyl-1,4-naphthoquinone will counteract about 1 gm. of acetyl salicylate (325). The failure of Lester (326) to find salicylates in the urine of rats fed 3,3'-methylenebis (4-hydroxycoumarin) seems to indicate that the latter is not hemorrhagic because of a breakdown to salicylates.

#### OTHER FACTORS

Further work on the antistiffness factor (133) indicates that a deficiency of this factor produces a muscular dystrophy which is not accompanied by creatinuria (327). This serves to distinguish the condition from that produced by avitaminosis E.

Scharf & Slanetz (328) have confirmed their earlier findings that soybean lecithin contains an unknown factor essential for the utilization of vitamins A and E. This is at variance with the view of Jensen and co-workers (329) that the vitamin A enhancing property of soybean phosphatides is due primarily to its tocopherol content, but it is in essential agreement with the results of Patrick (264). Patrick says that the soybean phosphatide factor seems to merely act as an antioxidant to protect vitamin A and enhance the antioxidative activity of vitamin E. Patrick made no claims for a new vitamin or vitamin-like factor.

A possible new vitamin of the fat-soluble group has been described by Bunzell (330) as a constituent of wheat-germ oil. The new factor speeds up the tyrosinase oxidation of *p*-cresol, a function not performed by vitamin A or vitamin E. Vitamin K does have this property, but it is ruled out as the active principle because of its extremely low concentration in wheat-germ oil.



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# THE CHEMISTRY OF THE HORMONES

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Chemical studies of the hormones have proceeded apace despite the handicap of the war, and now involve so many aspects of metabolism, clinical endocrinology, and enzymology that it is possible here to summarize only the highlights of recent studies. Fortunately during the past year three symposia on the subject have appeared: two under the auspices of the American Association for the Advancement of Science (1, 2), and the first volume of "Vitamins and Hormones" (3). These publications offer bibliographies comprising many hundreds of titles and will doubtless prove to be most useful source books.

## ANTERIOR PITUITARY HORMONES

*Differentiation of pituitary hormones.*—During the past two years important advances have been made in the preparation of pure proteins which have discrete physiological activities. The thyrotropic hormone from beef pituitary glands (4) and the adrenotropic hormone from both sheep and hog glands have been isolated in homogeneous states. Quite recently the growth hormone has been prepared in a high state of purity (5). In addition agreement has been reached on the physiological differentiation between lactogenic and growth hormones (6). Thus there are now at least six hormones in extracts of the anterior pituitary which have been identified as individual substances (7): lactogenic, adrenocorticotropic, luteinizing, growth, thyrotropic, and follicle-stimulating hormones. Of these, the first four have been isolated in chemically pure form. The thyrotropic hormone has only recently been highly purified and isolated (8). The follicle-stimulating hormone is available in a preparation free from other endocrine activity and is probably chemically distinct from the other hormones (9).

*Growth hormone.*—Li, Evans & Simpson (5) have recently described the isolation and properties of the anterior hypophyseal growth hormone. In this latest method, treatment with cysteine (10) to destroy thyrotropic, gonadotropic, and lactogenic activities was omitted.

Acetone-dried powder prepared from ox glands was the source. From a calcium hydroxide extract a globulin fraction was selected which was further fractionated with sodium chloride. Subsequent purification was effected by appropriate manipulations of the pH and precipitation with ammonium sulfate. Finally isoelectric precipitation was used.

Osmotic pressure determinations show the resulting purified protein to have a molecular weight of 44,300. Its isoelectric point is pH 6.85 by electrophoresis. Analytical data show sulfur 1.3, nitrogen 15.6, amino nitrogen 0.76, amide nitrogen 1.20, tyrosine 4.30, tryptophane 0.92, and glutamic acid 13.40 per cent. The number of acid groups per 10,000 gm. of protein is 9.80; of basic groups 13.40. The physiological activity is destroyed by pepsin and trypsin. The hormone is stable in urea solutions, but its activity is destroyed at pH 7 between 70 and 80° C. as the protein is coagulated. The hormone is more stable in alkaline than in acid media.

Li & Evans (7) report that this preparation when tested biologically gives no histological evidence of thyrotropic, adrenocorticotropic, or gonadotropic activity. Furthermore no crop response is elicited in squabs.

With this pure material available it will now be possible to decide whether the growth hormone is identical with the hypothesized diabetogenic, ketogenic, respiratory-quotient lowering, or myoglycstatic factors. Such physiological problems have plagued endocrinologists for many years.

*Lactogenic hormone.*—The lactogenic hormone (prolactin), which was crystallized by White, Catchpole & Long (11), was the first of the pituitary proteins to be isolated in discrete form. Of the various measurements made, the following characteristics are of interest: molecular weight, 32,000; isoelectric point, pH 5.7; minimum effective dose in the squab, 50 µg. The hormone is thermolabile. Of special interest is the higher tyrosine content of beef than of sheep prolactin (12). Treatment of the lactogenic hormone with urea increases the relative viscosity of the solutions with concomitant loss of potency. On dialysis, however, the hormonal activity is restored. After treatment with a suitable detergent irreversible loss of activity occurs (13).

One of the physiological clarifications which the purification of hormones has yielded is the differentiation of lactogenic and growth hormones. In pigeons, Riddle and his co-workers (14) produced body growth with combinations of thyrotropic and lactogenic preparations.

Therefore they questioned the concept of a growth hormone as an individual entity. It is now clear that pigeons respond differently than mammals. Growth hormone fails to produce a crop response in pigeons, whereas prolactin does favor body growth in pigeons. More data are needed on the reciprocal experiments before physiological separation of the two is complete, but already it is clear that the two individuals exist in fact.

The prolactin content of the human hypophysis has been assayed in both sexes and found to be approximately 0.3 units per mg. (2, 15).

*Adrenotropic hormone.*—The isolation of the adrenocorticotrophic hormone has recently been accomplished in two American laboratories (16, 17). Both groups started with the acid-acetone extract of fresh glands. Li, Evans & Simpson (17) used fresh sheep pituitaries whereas Sayers, White & Long (18) used glands from swine. The two preparations exhibited almost identical physiological activities when tested both by repair and maintenance effects. Both preparations gave a molecular weight of 20,000, an isoelectric point at pH 4.7, and a sulfur content of 2.3 per cent in the absence of cysteine. The material was stable in neutral solutions at 100° C., unlike all other pituitary hormones. At this temperature it was stable in acid solution, but rapidly destroyed in alkaline solution. It was rather stable toward pepsin but rapidly inactivated by trypsin.

Although this hormone retards the growth of male rats, an antagonism to such inhibition is produced by the growth hormone. Part of the inhibitory effect of adrenotropic hormone upon growth involves nitrogen metabolism. The increase in serum protein concentration, however, is probably due to hemoconcentration (19). Dougherty & White (20) have demonstrated that the involutionary effect of this hormone on thymus and lymphatic tissue may result in a transient leukopenia. Sturm & Murphy (21) have studied this effect on neoplastic processes involving lymphoid tissue.

Characteristic changes in the adrenals can be measured after known dosage of adrenotropic hormone. Marked decreases in ascorbic acid and cholesterol are noted (22). For bioassay, Simpson and collaborators (23) suggest two methods. The first depends upon histological examination of the adrenals; the second upon maintenance of the presumed preoperative adrenal weight.

*Thyrotropic hormone.*—Purified thyrotropic hormone preparations have been produced by Ciereszko & White (4, 24) from both sheep and beef glands. The preparation obtained from beef has an approxi-

mate molecular weight of 10,000, and is homogeneous in the Tiselius apparatus and in the ultracentrifuge. One microgram of this substance suffices to produce a definite effect in the chick. Certain preparations from sheep pituitaries, although contaminated with another protein, have shown a minimum effective dose (in the chick) of 0.5  $\mu$ g. The product is readily soluble in water. It contains 12.4 per cent nitrogen and 1.0 per cent sulfur. Phosphorus is absent.

After exposure to normal human thyroid tissue *in vitro* thyrotropic activity is markedly diminished (25). The effect is even greater with slices of tissue from human cases of Graves' disease. *In vivo* experiments (26) indicate that follicles stimulated by the tropic hormone are more basophilic. Milco (27) has assayed the hormone by measuring the height of the thyroid cells in infantile male rats.

*Gonadotropic hormones.*—In addition to prolactin two discrete hormones have been segregated, namely, the follicle-stimulating hormone (thylakentrin) and the luteinizing hormone (metakentrin). These have been discussed by Chow (9). Partially pure preparations of these two have been prepared and separated from each other by virtue of the lower solubility of metakentrin in organic and inorganic salt solutions. A detailed method of preparation of sheep pituitary gonadotropins with separation from the lactogenic hormone has been described by McShan & Meyer (28).

Greep, van Dyke & Chow (29) has prepared thylakentrin (FSH) from hog glands in biologically distinct form. Nevertheless physico-chemical study (30) has shown that this preparation is not homogeneous, even though the serum of rabbits immunized against hog metakentrin (LH) does not react with pure hog thylakentrin.

Preparations of metakentrin have been isolated in three laboratories (31, 32, 33) from swine and sheep pituitaries. All preparations have the same activity per unit weight. The preparations from sheep and hog differ in several important properties. Comparative values for preparations from sheep and hog are as follows: molecular weights 40,000 and 100,000; isoelectric points 4.6 and 7.45; carbohydrate 4.5 per cent and 2.0 per cent; and tryptophane 1 per cent and 3.8 per cent, respectively.

Both activities are destroyed by proteolytic enzymes, but metakentrin is more susceptible. Amylolytic enzymes like ptyalin or taka-diastase, however, destroy thylakentrin activity but not metakentrin. This finding (34) suggests that the follicle-stimulating substance contains one or more essential carbohydrate groups.

*Anterior pituitary-like (APL) gonadotropins.*—These hormones have been discussed recently by Gurin (35). The chorionic gonadotropin has lately been prepared from human pregnancy urine (36) by chromatographic adsorption upon permutite followed by elution of the hormone with 10 per cent ammonium acetate in 38 per cent aqueous ethanol, preferably at 0 to 10° C. Although such preparations are not quite homogeneous by electrophoretic studies, the molecular weight is probably approximately 100,000 (37). Such preparations contain 4,000 Friedman units per mg., an activity much greater than the homologous pituitary protein.

The chorionic protein contains over 10 per cent of galactose, over 5 per cent of hexosamine, and approximately 3 per cent of acetyl groups. These values correspond to mole ratios of 2:1:2. The actual identity of the polysaccharide moiety from the protein has not been determined. No prosthetic group has been identified. After hydrolysis Friedrich (38) obtained glucosamine from his preparation, which originally contained up to 24.0 per cent of carbohydrate and 7.8 per cent of nitrogen.

*Equine gonadotropin.*—This substance has been prepared from pregnant mare serum by fractional precipitation of impurities with 60 per cent alcohol and 50 per cent aqueous acetone near neutrality. Li and co-workers (39) have found such preparations to consist of at least two components. The minimal molecular weight of 30,000 calculated from the tryptophane and tyrosine contents, therefore, is in doubt. The material is a mucoprotein containing galactose and hexosamine in a mole ratio of 2:1. The galactose concentration is 17.6 per cent. The isoelectric point is near pH 2.63. Such material contains 4,000 to 7,000 I.U. per mg. Inasmuch as the hormone is rapidly inactivated by salivary enzymes and by takadiastase (35) it seems likely that the intact carbohydrate moiety is needed for maximal activity. Evans & Hauschildt (40) report inactivation by trypsin, pepsin, and also by high acidity.

A preparation containing 12,500 I.U. per mg. prepared by adsorption of the active material on benzoic acid has been described by Rimington & Rowlands (41). The active fractions are thermolabile at 60° C. and unstable in aqueous solution at 37° C. The glycoprotein contains hexose and hexosamine in a ratio of 2:1.

Although chorionic and equine gonadotropins are similar in several chemical properties, the physiological significance of this similarity is not clear.



## POSTERIOR PITUITARY HORMONES

Recent work on the posterior pituitary has centered about the problem of whether there are one or three essential constituents. The question has been discussed by Irving (42). Originally Kamm (43) found pressor and oxytocic activities in preparations consisting of small molecules with a molecular weight of about 600. It was recognized that these might represent split products of a larger molecule. Recently van Dyke and his co-workers (44) have obtained evidence supporting the unitary principle of Abel. They isolated a protein which is homogeneous and which possesses pressor, oxytocic, and antidiuretic activities in the ratio of 1:1:1. This "mother-molecule" is considered to be the storage form of the three sub-hormones. Its molecular weight is 30,000. It shows a solubility curve typical of a pure substance with an isoelectric point at pH 4.8. From this pure protein cleavage products can be obtained and separated. Such fractions resemble those obtained by the usual methods directly from the gland. They constitute separate polypeptides having molecular weights between 600 and 2,000. The cystine sulfur is 4.3 per cent, indicating that there are approximately twenty cystine molecules in the mother protein. These peptides have the high potencies of 450 pressor units per milligram and 700 oxytocic units per milligram. The original protein contains only 17 units per milligram of each of the three biological properties. Inactivation of oxytocic activity results rapidly from trypsin but only slowly from pepsin digestion.

## THYROGLOBULIN AND INSULIN

*Iodine metabolism of the thyroid gland.*—Because of the presence of iodine in the thyroid, considerably more progress has been made with this hormone than with insulin. Iodine determinations upon whole thyroid tissue and various subfractions thereof under a wide variety of conditions continue to appear. Thus the iodine in beef and hog thyroids from the south of India (45) was found to be approximately 0.8 per cent of the dry weight, a value considerably higher than that found in thyroids of North American animals. Wilmanns (46) reports the presence in fetal glands of organically bound iodine which is not protein in character. Interest in the various iodine compounds within the gland has stimulated many studies. For example, Leblond *et al.* (47) found that after exposure to cold the thyroids of rats fix 2.7 times as much radioactive iodine as do the thyroids of the control animals.

Separation of the substituent chemical fractions indicated that the excretion of iodized products by the gland occurs at twice the normal rate under this stress.

The availability of several radioactive isotopes of iodine (48) has facilitated studies on small amounts of tissue. Recently Salter & McKay (49) have described microcatalytic procedures applicable to the glands and blood of small animals. The combination of such microchemical procedures with the radioactive technique is highly desirable; first, because the partition of various iodine-containing fractions can be determined, and secondly, because through combined techniques it is possible to guard against the errors introduced by atomic "exchange reactions." This hazard has been discussed by Schoenheimer (50). Recent work (51) suggests that under controlled conditions, such as the avoidance of marked acidity, the difficulty can be obviated in the case of iodine. The question, however, is still moot and demands further careful study because radioactive iodine is being employed extensively in critical investigations of the intimate metabolism of the thyroid (52).

*Biosynthesis of thyroxine.*—The mechanism of the biosynthesis of thyroxine has interested several workers in recent months and years. In his Croonian Lecture Harington (53) has posed several of the problems involved in this question. These problems also include the appearance of thyroxine in the evolutionary scale as pointed out by Means (54).

The original hypothesis of Harington & Barger (55) that thyroxine was probably formed by coupling of two molecules of diiodotyrosine presupposed the loss of one side chain. That thyroxine could indeed be formed directly from diiodotyrosine was shown by Mutzenbecher (56) who incubated the former substance in alkaline solution for long periods.

Johnson & Tewkesbury (57) proposed an oxidative process as the mechanism by which this reaction proceeded. Recently Harington (58) has developed conditions whereby diiodotyrosine is oxidized directly to thyroxine by hydrogen peroxide in alkaline solution. He assumes that the phenoxide ions of the type of diiodotyrosine (as it exists at the pH of the body tissues) yield a diphenyl ether. This reaction is actually favored by iodine atoms in positions *ortho* to the phenolic group. What the oxidizing system might be within cells is a point for further conjecture. Schachner, Franklin & Chaikoff (59) have used radioactive iodine to study iodine metabolism in surviving

thyroid tissue. They found that iodine is fixed in organic combination by whole slices of the thyroid tissue. Homogenized tissue will not produce the reaction. Furthermore, the reaction does not occur under complete anaerobiosis nor if the utilization of oxygen is inhibited by such typical inhibitors of cytochrome oxidase as cyanide, azide, or hydrogen sulfide. Keston (60) has shown that the Schardinger enzyme (from milk) can facilitate the formation of organically bound iodine in crude preparations which contain xanthine oxidase, a peroxidase, and casein. The reaction is inhibited by thiourea. Franklin, Lerner & Chaikoff (61) have also used *in vitro* such goitrogenic agents as thiocyanate and thiourea. The former drug prevented the fixation of iodine by the tissue; the latter group permits fixation but only at the inorganic stage.

These several observations with radioactive iodine constitute suggestive evidence that the natural series of successive syntheses follows the same pattern as that previously developed in the laboratory. In these various observations the assumption has been made that free molecules of diiodotyrosine constitute the starting material for this natural synthesis. Salter (62) has suggested that the manufacture of thyroglobulin may occur independently of iodine metabolism. In this case the preformed protein would constitute a scaffolding upon which free iodine or an equivalent enzyme system might operate. Thus the successive stages of hormone synthesis would yield the storage form, aggregated in molecules of colloidal dimensions. Dempsey (63) has observed changes in autofluorescence and certain histochemical reactions at different states of physiological activity.

Other studies have been made on thyroid metabolism with surviving tissue slices. Chaikoff (64, 65) and his associates have continued studies on the accumulation of radioactive iodine by slices of thyroid. This iodine is subsequently incorporated into organic fractions resembling the natural diiodotyrosine and thyroxine moieties. The process is inhibited by high concentrations of iodide. Sulfanilamide and sodium azide inhibited the conversion of inorganic to organic form. Apparently iodine may be concentrated in the gland without being incorporated in thyroglobulin or its constituents. Similar studies have also been performed with tissues other than thyroid both by Chaikoff and by Chapman and his associates (66). Both laboratories suggest that body tissues other than the thyroid may retain a primitive ability to synthesize a thyroid-like metabolite.

Recent studies by Reineke, Williamson & Turner (67) and their

associates have improved the yield of potent material obtained from iodinated casein. Elevation of temperature has contributed toward more efficient synthesis. Such modified protein apparently has all the activity of the natural thyroid hormone. In appropriate amounts it produces characteristic changes in the growth and feathering of young chicks (68). The material is not excreted by the mammary glands of lactating cows (69). Pure *d*- and *l*-isomers are available (53).

*Activity of isomers of thyroxine.*—Already many congeners of thyroxine have been synthesized by Harington (70), Niemann (71) and Block (72) together with their respective associates. The net results show that a high specificity of structure is required to produce significant activity. Substitution of the iodine atoms in unusual places (e.g., at positions 4', 6') strikingly reduces activity. So also does the distortion of the thyronine nucleus, as shown by Bovarnick and associates (73) who blocked the terminal phenolic hydroxyl with a third diiodophenolic group. Such findings are useful in defining and limiting the problem of natural synthesis.

*The circulating hormone.*—Reports on human plasma (74) continue to indicate that the protein-bound iodine in circulation is a reliable index of thyroid activity (65). Because there are striking differences between the chemical behavior of hyperthyroid plasma and myxedematous plasma reinforced to an equal iodine content with pure thyroxine (75), many investigators have speculated upon the precise state of the circulating organically bound iodine. Salter, Oncley & Wheeler (76) have recently examined purified protein fractions from pooled human serum supplied by E. J. Cohn (77). These recent observations corroborate earlier findings by Bassett, Coons & Salter (78) who obtained a rough iodine spectrum for human plasma. The major part of the circulating iodine resides in the albumin fraction, but the highest concentration of iodine was found in the alpha and beta globulins. By special microtechnique this "hormonal" iodine, designated by Salter (75) as "P"-iodine, could be separated into "T" and "D" moieties. In myxedema the "hormonal" iodine is only approximately 1  $\mu$ g. per cent and the "T" moiety is absent. In severe thyrotoxicosis the "hormonal" iodine may approach 20  $\mu$ g. per cent and the "T" fraction may constitute three fourths of it. Thus it would appear that the thyroid hormone circulates as an integral part of the plasma protein, much as do antibodies. That thyroglobulin itself is not present in the serum was demonstrated by Lerman (79) who developed a highly sensitive test employing immune serum. Indeed

Meyer *et al.* (80) have recently shown that in rabbits such immune serum can cause a myxedematous state.

This work is consistent with studies by Clutton, Harington & Yuill (81) who attached to serum proteins residues of N-carbobenzyloxy-3,5-diiodothyronine, which were then iodinated. Such preparations were highly antigenic and would provoke a strong antibody reaction. The antisera could passively immunize an animal against thyroid hormone whether administered as thyroxine or as thyroglobulin. These observations collectively exclude thyroglobulin itself as the circulating hormone, except immediately after partial thyroidectomy or other trauma.

The capacity of hyperthyroid blood to elevate the oxygen consumption of surviving mammalian tissues has been demonstrated by Craig, Cortell & Salter (82). The tissue preparations show a decline of oxygen consumption while the thyroid effect is building up over the course of several hours. Consequently one can demonstrate an effect only by selecting conditions which retard the disintegration of intracellular mechanisms of the excised tissues.

*Goitrogenic agents.*—Renewed interest in the internal mechanisms of thyroid physiology has been stimulated by recent work on sulfanilamide derivatives (83) and thiourea substitution products (84). Astwood and his co-workers (2, 85) have studied several hundred chemical compounds, but at the present writing none has been reported with a greater therapeutic index than thiouracil. The mechanism of action still remains obscure but certain suggestive facts have been established. Such thyroid enlargement does not occur in hypophysectomized animals. The hyperplasia is prevented by giving thyroid or thyroxine. The goitrogenic agents do not nullify the action of exogenous thyroid hormone. These facts indicate that the hyperplasia is secondary to stimulation by the thyrotropic hormone from the anterior pituitary.

A rather wide variety of chemical compounds was found to be goitrogenic, but most of them can be fitted into two categories. The first of these is thiourea derivatives including the following: 2-thiouracil, 2-thiobarbituric acid, *sym.*-diethylthiourea and 5-benzal-2-thiohydantoin. The second comprises such aniline derivatives as the sulfonamides, *p*-, *m*-, and *o*-aminobenzoic acids, *p*-aminophenylacetic acid and *p*-aminoacetanilide. Although it is known that unusual concentrations of iodide will antagonize the goitrogenic action of thiocyanates, it is not yet known to what extent this antagonism exists in the case of other compounds. Thiouracil goiter is prevented by *l*-thyroxine.

Astwood & Bissell (86) found that after withdrawal of the drug,

injection of thyroxine or removal of the pituitary retarded the accumulation of iodine. Such glands became filled with colloid which contained very little iodine. It was formerly believed that pharmacological doses of iodine reversed the effect of thiocyanate, but not that of thiouracil. This feature, however, appears to be the result of a relative dosage whereby iodine and the goitrogenic substance act as if mutually antagonistic. The actual mechanism involved is uncertain, but Astwood has demonstrated that *in vitro* thiouracil reduces iodine to iodide and Dempsey (87) has demonstrated that a peroxidase present in thyroid cells tends to disappear when thiourea is administered.

Rawson, Tannheimer & Peacock (88, 89) and their associates observed that in rats the average uptake of tracer doses of radioactive iodine by the thyroid was markedly diminished by thiouracil, whereas thiocyanate increased the uptake. Similar findings were reported by Franklin, Lerner & Chaikoff (61). The latter group showed also (90) that these drugs prevented the fixation of iodine by surviving sections of thyroid. Larson *et al.* (91) have compared the effect of thiouracil with comparable stimulating doses of thyrotropic hormone. Williams *et al.* (92) demonstrated that anatomical changes in other organs were insignificant. This group failed to demonstrate a definite correlation between the concentration of circulating drug and the effect on thyroid function.

The goitrogenic substances prevent the formation of diiodotyrosine and thyroxine both *in vivo* (93) and *in vitro* (94). The result in young rats is ultimate cretinism (95). The marked hyperplasia of the gland can be prevented by simultaneous administration of exogenous thyroid hormone and this counteraction can be used (96) as an assay procedure. Thiouracil is actually stored in the thyroid (97) and this storage is decreased by thyrotropic hormone and increased by potassium iodide.

Many other studies of the effect of these goitrogenic agents are appearing and will doubtless shed much light on the physiological chemistry of the gland. However, space does not permit citing all the observations.

*Mechanism of action of thyroxine.*—Recent work by the Hoffmanns (98) has demonstrated that in hyperthyroidism induced in rats by thyroxine, a curious block of the vagal (i.e., muscarinic) effect on the heart occurs. In consequence, vagal action causes the heart to accelerate. The phenomenon is somewhat analogous to the well known action of ephedrine and possibly of prostigmine.

*Insulin.*—As pointed out by Jensen (99) the chemistry of insulin has not been advanced greatly in the past few years. Waugh (100) has studied the thixotropic gels derived from insulin hydrochloride and finds that they contain uniform fibrils several micra in length, with uniform widths of approximately 200 Å. He suggests that insulin molecules are linked mainly through the secondary valence forces resulting from the approximation of nonpolar side chains. Eisenbrand, Sienz & Wegel (101) have studied the zinc content of the human pancreas and found concentrations ranging from 18.5 to 30.4 mg./kg. of fresh gland. They showed by electrometric titration that the complex formed by zinc and insulin is analogous to that formed by zinc and glycine. The zinc content of insulin varies with the preliminary treatment of the protein. Possibly insulin combines with zinc maximally in amounts equivalent to its carboxyl groups. Saturation values as high as 3.5 per cent have been found with amorphous insulin, but after the process of crystallization this value does not exceed 1.73 per cent. Indeed Cohn and his co-workers (102) employing radioactive zinc found that the amount of metal varies from 0.3 to 0.6 per cent, depending upon the pH of crystallization. These authors found its molecular weight to be 46,000, whereas Miller & Andersson (103) reported 36,000. The latter authors found that when the sulfur of insulin is reduced with thioglycolic acid, molecules are aggregated to form particles of much greater size. Thus far all attempts to define a prosthetic group in the insulin molecule have failed. Most chemical substitutions result in diminished activity. For example Reiner, Keston & Green (104) obtained azo compounds in crystalline form. When such azo groups contained negative substituents, they produced less impairment of physiological activity of the parent protein.

Wasserman & Mirsky (105) found that insulins derived from various species were immunologically identical. Apparently future advance in this field will depend upon new procedures for determining protein structure. Lerman (106) found that antibodies to insulin are antihormonic, and that resistance to insulin is dependent upon the bodily concentration of such antibodies.

In totally pancreatectomized patients the insulin requirement is very small (107), a finding which suggests that the pancreas may be able to remove or destroy circulating insulin. Such resistance must be distinguished from the effect of delayed absorption, which may be secondary to it. Insulin labeled with radioactive iodine has been employed in human diabetics (108) to demonstrate this point. Attempts

are still being made to devise a preparation of insulin suitable for oral use. The injection of insulin directly into various parts of the gastrointestinal tract of rabbits (109) has shown that it is absorbed from the lower duodenum and from the jejunum and ileum.

#### ANTIHORMONES

This field has been reviewed recently by Thompson (110) but is nearly static. It still remains undecided whether such substances are to be regarded as (a) antibodies, (b) chalone, or (c) specialized enzymes. The work of Chow (30), already cited in connection with thylakentrin and metakentrin, has indicated that such antihormones can be both hormone specific and species specific. This work therefore differs from the results of Singer (111) who found that pituitary proteins would produce antibodies against kidney protein. Similarly van der Ende (112) reported that mare chorionic hormone provoked antibodies against seroglycoid.

That the host animal is important is evident from work by Marine and associates (80) who successfully used purified human thyroglobulin to produce antibodies in rabbits but failed to do so in rats. It is interesting that the rabbits eventually became myxedematous. Of practical significance are the studies of Leatham (113) who has observed clinical patients under therapy with various modern endocrine preparations. Of special importance is his finding of the development of antibodies against gonadotropin derived from pregnant mare serum when administered repeatedly for several months. Sufficient antibodies may be present to inhibit considerable doses of administered hormone. Certain anaphylactoid reactions have also been encountered. Some four months after cessation of therapy the antihormone disappears from the blood stream, but is rapidly regenerated when injections of purified pregnant mare serum extract are resumed.

In short, immune reactions have proved useful in identifying several of the purified hormones previously mentioned in this review. Although such antibodies may interfere with clinical therapy, there is as yet no clear evidence that this class of substances is an integral part of natural physiological mechanisms.

#### ESTROGENS

Recent chemical studies of estrogens have centered chiefly about two main topics: first, the excretion of steroids in urine; and second, the intermediate metabolism of the sex hormones. The first of these



topics has been reviewed by Gallagher (114); the second by Pincus & Pearlman (115). In addition, there has been a host of physiological studies based upon chemical techniques so that any selection must be arbitrary. Various new chemical techniques have also appeared but of these only a few can be mentioned. A historical review on the discovery and isolation of the female sex hormones has been prepared by Newerla (116).

*Estrogenic hormones in urine.*—Several new methods have appeared for the determination of urinary estrogens. Anhydrous aluminum oxide has been used for the chromatographic adsorption of naturally occurring estrogens with a recovery of from 80 to 100 per cent (117). With appropriate mixtures of benzene and methanol as eluting agents the strongly phenolic (estriol) fraction can be separated from the weakly phenolic (estrone and estradiol) fraction. An electrophotometric modification of Kober's method has been described in which acetone is added to the colored reaction mixture (118). The initial reading gives folliculin plus other pigments. On standing overnight the folliculin color fades. The Pulfrich step-photometer has also been utilized (119) for the chemical determination of urinary estrone and estriol in both free and bound forms. Curtis, Witt & Knudsen (120) have made a statistically evaluated study in rats of a technique for the assay of estrogen preparations. This procedure should prove valuable for the control of chemical methodology. For the determination of total estrone and estradiol from tissue sources Szego & Samuels (121) described a colorimetric reaction with potassium guaiacolsulfonate. The estradiol fraction was separated by the Girard method (122). A curious indirect index of estrogenic activity in women, based on the refractive index of cervical secretions, has been suggested (123), but this test should be used with great caution.

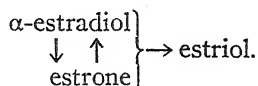
There is still considerable doubt as to the best procedure for the preliminary extraction of estrogenic substances. Doisy (124) has pointed out that more data are needed on the completeness of hydrolysis and the degree of destruction in studies of the phenolic estrogens. In monkey urine after the routine extraction a further yield approximately equal to the first extract was obtained. Depending upon how drastic the conditions of extraction are, various degrees of hydrolysis of naturally occurring esters are encountered. Thus after the parenteral administration of stilbestrol to rabbits Mazur & Shorr (125) isolated the monoglycuronide. In a rather similar experiment Bass & Salter (126) obtained only free stilbestrol because the condi-

tions of the extraction were more drastic. A further complication is that both the  $\alpha$  and the  $\beta$  epimers of estradiol may be excreted together in the urine under certain conditions. Pearlman & Pearlman (127) reported that the estradiol fraction of urine from rabbits injected with estrone consists almost exclusively of the  $\alpha$ -isomer: whereas the homologous fraction of urine from rabbits injected with estrone consists almost exclusively of the  $\beta$ -isomer. Other phenolic substances may complicate the final colorimetric determination. For example, Campbell & Hey (128) extracted ten gallons of stallions' urine and isolated approximately twenty-five grams of *p*-cresol together with some 680 mg. of estrone.

If a biological assay is applied to a chemical fraction from urine the result may be in error because some physiological effects attributed to estrogens are actually due to their metabolic products. For example, Smith (129) found that the so-called lactone isolated by Westerfeld (130) after treatment of estrone with hydrogen peroxide could stimulate the gonadotropic factors in the rat pituitary. Furthermore, combinations of various estrogens are hazardous when assayed biologically: one report (131) on combined estrone, estradiol, diethylstilbestrol, and 3,4-*p*-hydroxyphenylhexanediol dipropionate stated that the effects were additive or slightly greater than the estimated additive effect; whereas another report (132) on the comparative strength of dinestrol, hexestrol, and stilbestrol found that there was no mathematical basis for such a comparison, because their dosage-response curves are not parallel.

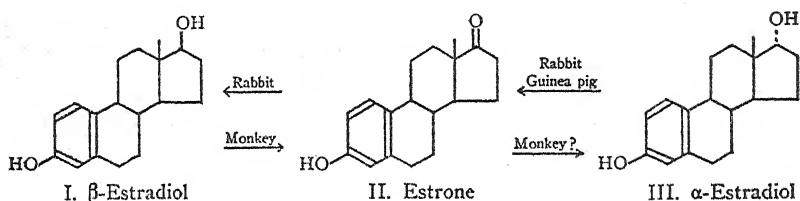
The problem of conjugation extends also to human pregnancy serum in which 23 to 50 per cent of the estrogens are conjugated. None of the estrogen passes through a collodion membrane (133), and all of the estrogen is removed when the plasma proteins are precipitated.

*Intermediary metabolism of estrogens.*—Doisy, Thayer & Van Bruggen (124) have observed that considerable variation in metabolism may exist from species to species. No recent evidence, however, has turned up to controvert the soundness of the following scheme in the human organism:



Possibly the further degradation of estriol may proceed through oxidation in rings A and D. A small portion may become saturated and ex-

creted as neutral estranediols (134). Szego & Samuels (135) studied the endometria of bovine, pregnant human, and pregnant rabbit. They found that the rabbit endometrium converted estrone aerobically nearly completely to estradiol, but that no significant change occurred with the bovine or the pregnant human endometrium. Species difference has led in part to contradictions in the literature. The most likely interrelationship which can be stated at the moment is the following:



After the intraperitoneal injection into guinea pigs of such pro-estrogens as triphenylethylene, and phenylstilbestrol, Emmens has demonstrated the excretion of urinary estrogen (136). The results were controlled by analogous experiments involving vaginal administration. As yet no detailed study is available on the qualitative identity of the excreted compounds.

*Estrogens and the liver.*—It has been known for over a decade that the liver contains a cyanide-sensitive enzyme system which can inactivate  $\alpha$ -estradiol and estrone. Heller & Heller (137) conclude from *in vitro* observations that estrone is first converted to  $\alpha$ -estradiol and that this step is not affected by cyanide, as is the subsequent oxidative destruction. It is reported (138, 139, 140) that the synthetic estrogens are more resistant to such hepatic destruction.

Cantarow and his associates (141) have suggested that there is an enterohepatic circulation of estrogens because considerable estrogenic activity is found in the bile under various conditions. Furthermore they deprecate the importance of inactivation (as opposed to excretion) because poisoning by chloroform produced no demonstrable loss of hepatic activity in this respect. On the contrary Schiller & Pincus (142, 143), from work with partially hepatectomized rats, conclude that hepatectomy interferes chiefly with the conversion of estrone to estrinol, because the excretion of the former is increased six-fold. Following the injection intravenously into dogs of estrone,  $\alpha$ -estradiol or stilbestrol, the circulating estrogen is maintained longer at high levels

in the presence of liver damage (144). Engel (145) finds that no significant inactivation of progesterone by the liver takes place. Various pathological consequences of this hepatic action have been studied including a hepatic autodefense against the tumorigenic action (146) of estrogens. Parenthetically, lymphoid tumors have been produced in mice receiving steroid hormones (147).

The activity of various synthetic compounds and preparations thereof continues to be reported. Rauscher (148) has compared the minimal dosage producing estrus in rats for the following compounds of diethylstilbestrol, and found resulting potencies as indicated: dipropionate, hydroxydipropionate, hydroxydiacetate, and dicarbethoxylate > monoglucoside pentaacetate > monomethyl ether > monomethyl ether propionate. These were all less active than the free diethylstilbestrol, which however barely exceeded hexestrol dipropionate in potency. Parkes (149) has studied in the capon the relative absorption of various aliphatic esters of estrone. From the propionate through the laurate ester the rate of absorption increased. For estradiol, the 17-propionate had low and the dipropionate high activity, and the 17-caprylate and the 3-benzoate-17-butyrate had activity similar to that of estrone palmitate. Perlingual assimilation of estradiol benzoate is half as efficient as absorption of the free hormone (150, 151) and equal to intramuscular medication. The caprylate derivative is said to be effective both orally and parenterally, and less toxic than diethylstilbestrol at comparable therapeutic levels (152).

The dipole moments of several androgens have been studied by Kumler & Fohlen (153), to learn whether physiological action is related directly to physical properties. The dipole moments of some of the compounds studied were as follows: androsterone, 3.70;  $\beta$ -androsterone, 2.95;  $\Delta^5$ -androstene-3( $\beta$ ),17( $\beta$ )-diol, 2.69; testosterone, 4.32; *cis*-testosterone, 5.17. No correlation could be demonstrated between the dipole moments of the sex hormones and their physiological activity.

*Lutein derivatives.*—The preparation and properties of relaxin have been described by Abramowitz, Kleinholz & Hisaw (154). From a methanol extract of frozen luteal tissue a grayish white solid was removed by salting out. Castrated female guinea pigs were used as test animals, in which relaxation of the symphysis pubis was determined. The test material, containing 15 per cent nitrogen, showed no estrogenic effect in rats following the injection of 120 relaxative doses and no progesterone effect in rabbits following 500 relaxative doses. This

effect therefore is highly suggestive of another distinct hormone in the corpus luteum.

Allen & Ehrenstein (155) reported the preparation of a mixture of stereoisomers of 10-*nor*progesterone from strophanthin. The ultra-violet absorption spectrum indicated an  $\alpha$ ,  $\beta$ -unsaturated ketone, with an absorption maximum at 238.5 millimicra and extinction coefficient of 16,560. This substance was approximately as active as progesterone in rabbits.

Pregnanediol, the excretion product of progesterone, appears in the urine of normal women as the glucuronide (156) and in rabbit urine after the administration of progesterone. Similarly desoxycorticosterone is excreted as urinary pregnanediol glucuronide when administered to man or to rabbit (157). Consequently more colorimetric tests for urinary pregnanediol are appearing. Jayle, Crepy & Wolf (158) determined pregnanediol glucuronide colorimetrically after a single extraction with butyl alcohol. A semi-quantitative color reaction with concentrated sulfuric acid has been applied by Guterman (159) as a test for human pregnancy. Preliminary extraction is made with acidified toluene, followed by treatment with alkaline methanol and acetone. Talbot and his associates (160) point out that during acid hydrolysis a 30 per cent loss may occur but this loss can be avoided through enzymic hydrolysis. The source of the enzyme is rat liver paste treated with acetone.

*Metabolism of progesterone.*—Both progesterone and anhydrohydroxyprogesterone when administered orally in man yield pregnanediol glucuronide (161). In rabbits this conversion occurs both in male and female (162), but neither uterus nor testis is essential. After doses of 30 mg. of progesterone, castrated women and post-menopausal subjects excrete less than 1  $\mu$ g. of unchanged hormone (163). Of the biologically inactive decomposition products excreted in the urine, 45 per cent is pregnanediol. In the last six months of pregnancy (164) average women excrete between 6 and 31 mg. of pregnanediol daily. This amount can be increased during the early months by single daily doses of 200 mg. of glucuronic acid. Portes, Simmonnet & Robey (165), however, failed to find pregnanediol in human urine after injecting 120 mg. of desoxycorticosterone. A comparison of progesterone and pregnenolone (166) indicates that characteristic changes in the sodium-potassium ratio produced by the former (given subcutaneously) are not uniformly produced by the latter (given orally).

Corner (167) has studied the sublingual absorption of various

steroid hormones in rhesus monkeys and in rabbits. In these animals the sublingual administration of progesterone, dissolved in mixtures of alcohol and propylene glycol, was unreliable. The dosage was over four times the intramuscular dose. This is true also of desoxycorticosterone, androgens, and pregnenolone, whereas estrogens tend to be equally effective whether by sublingual or intramuscular route.

Almost no evidence is available as to the biochemical mechanisms influenced by estrogens. In a recent study of tissue aldehydes, Oster (168) has found a change in the intercortico-medullary zone of the rat kidney concomitant with the estrous cycle. During estrus the aldehydes tend to increase but they decrease during diestrus.

### ANDROGENS

*The separation and determination of androgens.*—The major problem in the field of androgenic substances is their chemical differentiation from steroids of adrenal cortical origin. The problem is complicated by the finding (169, 170) of certain steroids which behave unlike the known 17-keto and 20-ketosteroids in that they are biologically active before hydrolysis. Perhaps they are excreted in the unconjugated form. Because these extracts maintain the life of adrenalectomized rats and favor the deposition of liver glycogen, it is suggested that the constituent steroids contain oxygen at C<sub>11</sub> and have an  $\alpha$ - $\beta$  unsaturated ketone in ring A. As yet these steroids have not been identified, although Wolfe, Fieser & Friedgood (171) tentatively described as  $\Delta^{11}$  androsterone a substance found in the urine of a patient with an adrenal tumor. They suggested that it might be a dehydration product of androstan-3,11-diol-17-one.

Because so many urinary 17-ketosteroids have been isolated by Dobriner and others (2, 172), attempts are being made to develop simplified methods which will distinguish various fractions more readily. Frame (173) has reported a micro method for the separation of  $\alpha$  and  $\beta$  fractions with recoveries of androsterone and dehydroisoandrosterone. This method involves precipitation by digitonin from the Girard ketonic fractions, with ultimate solution in dry pyridine. Cahen & Salter (174) have compared results obtained by a modified *m*-dinitrobenzene procedure with those yielded by the antimony trichloride reagent of Pincus (175). In general the results given by several steroids check well with the exception of dehydroisoandrosterone, toward which the Pincus reagent is relatively insensitive. Former reports that the Pincus reagent gave lower values than the older Zim-

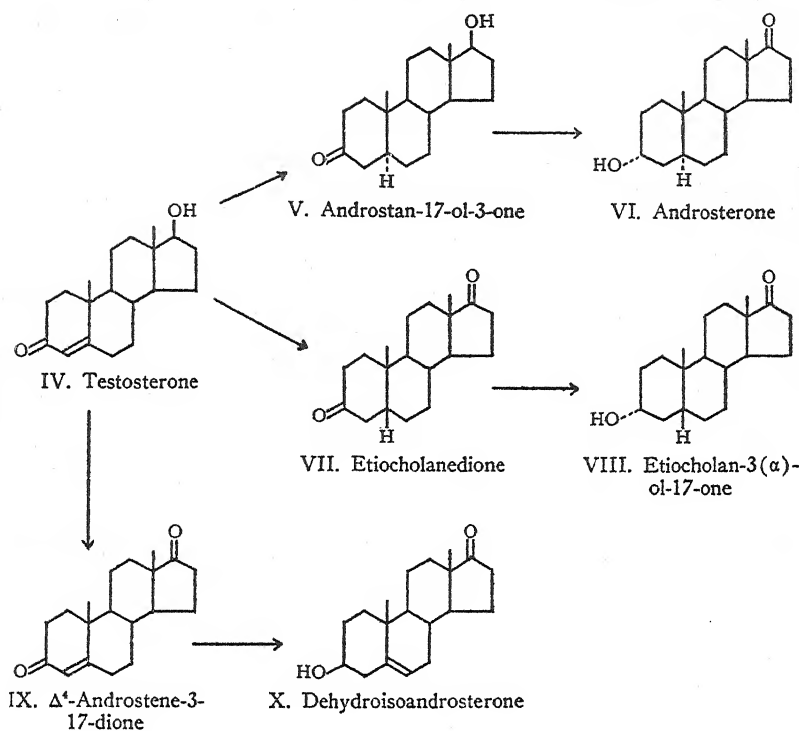
mermann technique were due to the fact that the older Zimmermann color was too high because of chromogen contributed by the reagent. In further work by Cahen and his associates (176) the differential color developed by the two respective reagents was tested as a means of distinguishing dehydroisoandrosterone. Progress in the same direction has been made by Kerr & Hoehn (177) who studied a modified Pettenkofer reaction. The work has been extended by Gallagher & Munson (114) who applied the Gregory-Pascoe reaction to a series of steroids yielding color maxima in the neighborhood of 660 millimicra. Dirscherl & Zilliken (178) have described a blue-violet (maximum at 500 millimicra) pigment formed with sulfuric acid by dehydroisoandrosterone and by *i*-androstan-17-ol-6-one. The reaction is not given by androsterone, testosterone, androstenedione, estrone, estradiol, progesterone, corticosterone, or cholesterol.

Friedgood, Taylor & Wright (179) have compared combined versus independent hydrolysis and extraction of urinary 17-ketosteroids. They recommend acid hydrolysis followed by ether extraction and subsequent toluene extraction of the residue from the ether solution. This procedure gave highest recoveries and less daily variation than two other combinations studied.

*Metabolism of testosterone.*—One of the chief objectives of present investigations is the determination of the intermediate metabolism of androgens. At present several alternate schemes for the degradation of testosterone are under consideration. Formulae IV–X on the opposite page are such, modified from Gallagher (114).

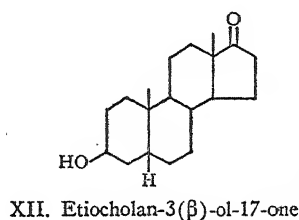
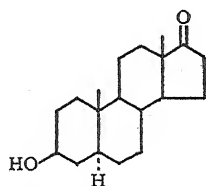
The inclusion of androsterone (VI), etiocholan-3-( $\alpha$ )-ol-17-one (VIII) and of dehydroisoandrosterone, i.e.,  $\Delta^5$ -etiocholan-3-( $\beta$ )-ol-17-one, (X), is due in part to their isolation by Callow & Callow (180) from the urine of a eunuch, and subsequently from the urine of normal men and men receiving testosterone therapy. In regard to dehydroisoandrosterone, Munson, Gallagher & Koch (181) have shown that the compound is not extensively metabolized. Therefore, it probably does not contribute the major portion of urinary androsterone (VI). At present it is difficult to describe the place of isoandrosterone, i.e., etioallocholan-3-( $\beta$ )-ol-17-one (XI), in the metabolic sequence. Furthermore, the fourth possible isomer, etiocholan-3-( $\beta$ )-ol-17-one (XII), has not been isolated to date.

An alternative suggestion was proposed by Dorfman & Hamilton (182), namely, that a certain amount of androsterone may originate by way of  $\Delta^4$ -androstene-3,17-dione (IX). Indeed this last substance



With respect to the 3-hydroxyl, compounds VI and VIII are  $\alpha$ (trans to  $-\text{CH}_3$  at position 10, 19). Compound VIII represents the *allo* configuration of the A and B rings. In this latter case, the use of *cis* is to be avoided. Cf. reference 217.

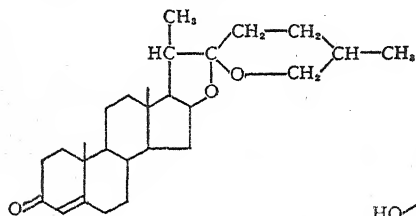
led to a greater excretion of androgen than all other substances tested except androsterone. It seems likely however that the major portion of the diketone is converted to dehydroisoandrosterone (X). This type of reduction has been studied by Marker *et al.* (183) who fed 4-dehydrotigogenone (XIII) to a dog and recovered diosgenin (XIV), together with two other steroidal derivatives. The finding suggests that



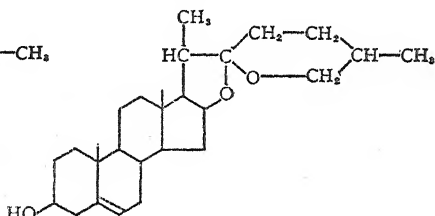


one of the first stages in the metabolism of testosterone (IV) is saturation of the double bond. The smaller portion which remains unsaturated may proceed to dehydroisoandrosterone (X). In other words, this last substance would constitute a by-product in the metabolism of ketones unsaturated in ring A, whether derived from the testis or the adrenal.

Because isolation experiments disclose androsterone (VI) and



XIII. 4-Dehydropregnenolone



XIV. Disogenin

3( $\alpha$ )-etiocolanolone (VIII) in approximately equivalent proportions, the inference is tenable that the reduction of the double bond in testosterone (IV) yields two isomers at C<sub>5</sub>. Moreover, in the *allo* series, although reduction of the ketone group at C<sub>3</sub> might occur either way, the fact is that the 3( $\beta$ )-hydroxy-steroid fraction is usually relatively small compared with the  $\alpha$ -steroids.

Obviously more data are needed concerning the intermediary metabolism of androgens. Krichesky and his associates (184) have shown that the androgens are acted upon by the liver. Cantarow and his associates (185) have also demonstrated in bile-fistula dogs the appearance of androgenic material in the bile following a single injection of androsterone, testosterone, or methyl testosterone. These findings are of special importance because a much greater proportion of the androgenic metabolism is unaccounted for than in the case of estrogens. It is interesting (186) that biochemical oxidation of gonadal hormones by *Micrococcus dehydrogenans* has been demonstrated. For example, dehydroandrosterone is oxidized to androstenedione almost quantitatively.

Various metabolic experiments designed to test possibilities similar to those just mentioned have been performed. Dehydroisoandrosterone sulfate has been isolated from normal male urine (187). In the pregnant rhesus monkey (188) the conversion of testosterone to androsterone has been demonstrated in experiments lasting seventy days. The recovery from 1.2 gm. of testosterone propionate, how-

ever, was only 22 mg. of androsterone. Control pregnant monkeys not receiving testosterone apparently produced no demonstrable androsterone. In male chimpanzees 1.5 gm. of testosterone propionate yielded 54 mg. of androsterone, and caused a 13-fold increase in urinary androgens (189). Of 1,050 mg. of 17-ketosteroids excreted, androsterone represented 5.1 per cent, androstenone-17, 11.2 per cent, and etiocholan-3( $\alpha$ )-ol-17-one, 2.0 per cent.

Little is known of the chemical mechanisms which are related to the origin of these hormones. The evidence that they may be derived from cholesterol is discussed elsewhere in this volume by Ruigh (190).

*Comparative biological effects.*—Indirect light on the intermediary metabolism of androgens may be gained from certain atypical biological experiments. In castrated female guinea pigs for example, abdominal fibroid tumors may be induced by  $\alpha$ -estradiol. This effect is inhibited by testosterone and progesterone (191) but not by 40-fold doses of  $\Delta^4$ -androstene-3,17-dione nor by cholestenone. After hypophysectomy in young male rats the following steroids protect the adrenal glands from atrophy: testosterone propionate; androstane-3( $\alpha$ ),17( $\alpha$ )-diol and  $\Delta^4$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol (192). No effect was demonstrated for the following: androstenedione, dehydroisoandrosterone, progesterone, and desoxycorticosterone. In young castrated male rats (193) desoxycorticosterone acetate caused enlargement of the prostate and testicles. Similar effects were produced by pregnenolone, desoxycorticosterone, and progesterone, in decreasing order of intensity. In mice (194) the weight of the kidneys was increased by testosterone propionate, but not by cholesterol, estradiol benzoate, progesterone and desoxycorticosterone acetate. In female mice, however, some effect has been reported from estradiol benzoate (195), but this effect does not occur in the case of males.

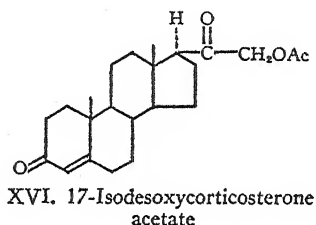
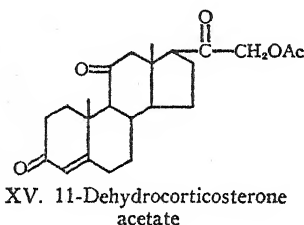
Selye (196) has proposed a "natural classification" of the steroids which considers the following functions: folliculoid, testoid, luteoid, corticoid, and anaesthetic. He has summarized the pharmacology of steroids with respect to these properties (2, 197).

A historical review on the discovery and isolation of the male sex hormones has been prepared by Newerla (198).

#### ADRENAL CORTICAL STEROIDS

*The origin and synthesis of steroid hormones.*—For many years it has been generally assumed as a working hypothesis that the steroid hormones are derived from cholesterol in the organism, although this

hypothesis has not been proved. As emphasized by Schwenk (199) and by Fieser (200), these hormones belong to a four-ring cyclopentanoperhydrophenanthrene system, which can exist in sixty-four isomeric forms because it contains six asymmetric carbon atoms. In the case of cholesterol alone, the eight asymmetric carbon atoms permit 256 isomers. Consequently any total synthesis *in vitro* is repeat-



edly complicated by the appearance of unwanted isomers, which plague the chemist at each successive step. Only in the case of equilenine has a total synthesis actually been accomplished, because only four isomers are possible in this naphthalene derivative. As shown by Ruzicka and his collaborators (201), oxidation of 5,6-dibromo-cholesteryl acetate yields as the main product dehydroisoandrosterone (X).

Accordingly it is convenient to think of the steroid hormones as derived from cholesterol. Schwenk (199) divides these further as follows:

A. Aromatic. These are the natural follicle hormones or estrogens. They include such natural substances as equilenine, hippulin, and equiline, as well as  $\alpha$ -estradiol (III), estrone (II), and estriol. They also include the artificial hormone ethynylestradiol.

B. Non-aromatic. These can be divided into hormones from the corpus luteum, the testis, and the adrenal cortex.

(1) The corpus luteum hormones include progesterone, and also the artificial hormone ethynyltestosterone.

(2) The androgens of natural origin are testosterone and androstenedione. The analogous artificial hormone is methyltestosterone.

(3) The hormones of the adrenal cortex collectively appear in the extract known as "cortin." The best known of these are corticosterone and desoxycorticosterone.

This simple outline serves to emphasize the interrelationship of the steroid hormones, which is important to the pure chemist and the biochemist. More elaborate presentations of this relationship have been

given by Pincus & Pearlman (115) and by Reichstein & Shoppee (202). How the natural synthesis occurs is still a matter of conjecture. Bloch & Rittenberg (203), however, found that after feeding acetic acid labeled with heavy hydrogen to rats or mice, cholesterol is obtained which contains the heavy hydrogen. The results are interpreted as indicating that cholesterol, at least, is synthesized from very small molecules. Whether the hormones are derived from cholesterol or synthesized *de novo* is still unknown.

The main facts concerning the isolation and identification of the various steroids of the adrenal cortex have been summarized recently by Kuizenga (204), who has described the preparation of mixed hormonal material equivalent to 2.5 grams of 11-dehydro-17-hydroxycorticosterone from 1,000 pounds of beef adrenal glands ground into 300 gallons of acetone. Nine grams of partially purified material obtained was further fractionated as follows:

- a. Benzene residues, 2.6 gm., containing corticosterone, dehydrocorticosterone, 11-desoxycorticosterone, and 11-desoxy-17-hydroxycorticosterone.
- b. Intermediate fraction soluble in benzene and water, 4.0 gm., containing 11-dehydro-17-hydroxycorticosterone.
- c. Aqueous residues, 1.2 gm., containing 17-oxycorticosterone.
- d. Resinous substance (separating during other procedures), 1.1 gm., containing compounds C and D of Reichstein.

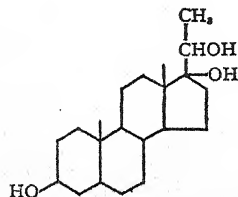
Of the chemical entities now recognized, the relative potency can now be stated for six crystalline compounds and an amorphous fraction (205). In the following synopsis the first value represents units per mg. as determined by the work test of Ingle (206), and is largely related to carbohydrate metabolism. The second value represents the survival growth test in rats, and probably reflects in large measure electrolyte metabolism. The values are: 17-hydroxycorticosterone 6.32, 10; 17-hydroxy-11-dehydrocorticosterone 5.00, 10; corticosterone 2.36, 8; 11-dehydrocorticosterone 1.66, 6; 11-desoxycorticosterone 0.1, 40; 17-hydroxy-11-desoxycorticosterone 0.1, 8; and for the amorphous fraction 0.6, 25.

The amorphous fraction is of special interest because of its high activity both in rat survival and dog assay methods. It is active when administered orally to adrenalectomized rats. It has very little progestational effect. Because the biological activity of adrenal extracts cannot at present be explained completely on the basis of known chem-

ical entities, it is hoped that new congeners will be isolated to explain the discrepancy. Recent extracts (207) prepared from beef, hog, and sheep glands indicate that the hog adrenal extract contains the most hormone both by the survival test and the muscle contraction test. It is suspected that the content of 11-oxygenated steroids in the hog extract is high.

*Analytical methods.*—Talbot and his associates (208) have contributed two useful procedures for the chemical determination of keto-steroids. The first of these is the analysis for dehydroisoandrosterone sulfate, based upon the finding that whereas hydrochloric acid hydrolysis destroys a major part of this material in aqueous media, the compound can be recovered quantitatively by hydrolyzing a washed *n*-butanol extract of the water or urine with barium chloride. The second method (209) involves the quantitative oxidation of 17,20-dihydroxy steroids to 17-ketosteroids with periodic acid. Apparently human urine contains steroids of adrenal cortical origin which are precursors of 17-ketosteroids. These precursors are destroyed by hydrolysis in hydrochloric acid but can be recovered after milder types of hydrolysis such as enzymatic or barium chloride procedures.

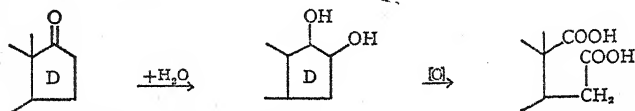
*Chemical structure in relation to synthesis and degradation.*—One of the problems which concerns the organic chemist and biochemist with reference to the chemical structure of steroidal hormones is the mechanism whereby these active molecules can be produced both in the organism and in the laboratory. Thus far it has been impossible to devise a practical synthesis of 11-oxygenated adrenal steroids, although the partial synthesis of the 11-dehydrocorticosterone (XV) has been accomplished by Lardon & Reichstein (210). Much important synthetic work, with special reference to stereochemistry, is now in progress. For example the importance of the 17- $\beta$  configuration has been demonstrated by the assay of 17-isodesoxycorticosterone (XVI) which is much less active than the normal desoxycorticosterone. Likewise



XVII. 3( $\alpha$ ),17,20-Pregnanetriol

various stages of degradation are being studied. For example it has been shown (202) that steroids like the 3( $\alpha$ ),17,20-pregnanetriol (XVII) of Marrian (211) can be oxidized readily to a 17-ketosteroid, and may acquire potency in the process. When progesterone is treated with lead tetraacetate a small yield of desoxycorticosterone acetate is produced (212).

How these various interconversions take place in the organism is the subject of various theories which have been recently reviewed (115, 202). One of the later theories concerns oxidative scission *in vivo* of ring D of the steroid molecule. This is based in part upon the isolation by Hirschmann (213) of a 3,16,17 steroid triol. According to the interpretation of Pearlman & Pincus (214) the following stages may occur:



The mechanism of conjugation by which the uronides are formed has been studied. Huebner and his associates (215) have produced synthetic pregnanediol 3( $\beta$ )-*d*-glucuronide, which is identical with the naturally occurring uronic acid conjugate of pregnanediol. They have also described the synthesis of pregnanediol 20-acetyl-3-(tetraacetyl- $\beta$ -*d*-glucoside) and stigmasterol triacetyl- $\beta$ -*d*-glucuronide methyl ester.

*Intermediary metabolism.*—New adrenal cortical steroids continue to be identified in the urine of normal and pathological human individuals. Dobriner and his associates (216) have performed extensive studies on the 17-ketosteroids in urines from normal individuals and cancerous patients, and have isolated compounds not previously obtained from normal urine. Wolfe and his associates (217) isolated androsten-3( $\alpha$ )-ol-17-one in a case of virilism with adrenal tumor. Marrian (211) has found  $\Delta^5$ -androstene-3( $\beta$ ),16,17-triol in the urine of a boy with adenocarcinoma. This compound is believed identical with the steroid described by Hirschmann (213) in a similar case. The conversion of desoxycorticosterone to 3( $\alpha$ ),20( $\alpha$ )-pregnanediol has been demonstrated (218) by direct isolation of the latter compound in human cases of Addison's disease, human eunuchoidism and an ovariectomized chimpanzee. That urinary cortin activity originates in the adrenal and not in the gonad seems proved by experiments in monkey and in man (219, 220) performed by Dorfman and his associates. The test animals were adrenalectomized rats to which ex-

tracts from the urine of appropriate donor animals or patients were administered orally. Collateral observations by Venning, Hoffman and Browne (221) indicate that after the stress of surgical operations human urine may contain three to thirty times as much cortin-like activity as normally. The post-operative urine was extracted with ethylene dichloride without hydrolysis. Finally the ketonic material was separated by Girard's Reagent T. This ketonic material when assayed by the Selye-Schenker test for protection against cold (222) contained between twenty-five and one hundred per cent of the activity of corticosterone.

The high turnover of steroids by the adrenal cortex has been described by Vogt (223), who estimated the cortical hormone in the venous effluent of the suprarenal gland of the dog, cat, goat, rabbit, and pig. The potency of one cc. of such plasma was as high as ten-fold that of an extract representing one gram of gland. The cortical hormone is rapidly inactivated in the tissues, but liver, spleen, kidney, and gastrointestinal tract are not essential for the process. Similar data have been obtained by Sayers and his associates (224) in studies of adrenal cholesterol. The concentration of this substance in the glands of hypophysectomized immature white rats diminished to less than 50 per cent of its normal level within three hours after the injection of pure adrenotropic hormone. Abelin (225) found that the adrenals are the site of an active metabolism of cholesterol which is influenced by many factors. These include pituitary extract, the feeding of pure saccharides, the administration of thyroid hormone and narcosis induced by ether, chloroform or a barbiturate derivative. This concept of high steroidal turnover is substantiated by the finding (226) of as much as 857 mg. of neutral 17-ketosteroids in twenty-four-hour urine samples of patients with tumor or hyperplasia of the adrenal cortex.

Many physiological studies involving chemical interrelationships continue to appear. These have been reviewed recently by Swingle & Remington (227). Ingle (228, 206, 229) has applied the increase in capacity for muscular work in a bioassay procedure which is rather specific for the  $C_{11}$  oxygenated cortical steroids. The relation of the adrenal steroids to pathological physiology also continues to hold great interest. Malignant hypertension (230) has been produced in rats treated with desoxycorticosterone acetate and sodium chloride. In human hypertensive individuals the urinary excretion of adrenal steroids is lower than in normotensive controls (231). In severely chilled guinea pigs (232) the hormone content of the adrenal cortex

undergoes a marked decrease. Exposure to low barometric pressure causes hypertrophy and hyperactivity of the gland (233).

Numerous biochemical studies of 17-ketosteroid excretion by normal and abnormal human subjects have appeared. Of these it is possible to cite only two, in addition to those already mentioned by Rhoads & Dobriner (172) and by Venning, Hoffman & Browne (221). Talbot and his associates (234) have studied the excretion by normal and abnormal children. It was found that the output of 17-ketosteroids is low from birth until approximately ten years of age. The values for older children gradually increase as the subjects approach eighteen years of age, the males steadily outstripping the females. By the twelfth birthday normal children should excrete at least 1 mg. daily. Children with somewhat precocious sexual development tend to have higher values, and the converse is true with those with delayed development. No consistent abnormality was noted among mongolian idiots. In the adult group Albright (235) has summarized recent studies with special reference to the role of the adrenal cortex under conditions of stress.

A water soluble preparation of desoxycorticosterone glucoside (236) is highly effective in adrenalectomized dogs. The effectiveness of desoxycorticosterone and desoxycorticosterone acetate in infantile female rabbits (237) has been demonstrated after both percutaneous and peroral administration. The test reaction employed was the secretory uterine phase after pretreatment with estrone. Based upon 6 mg. injected doses of the two substances named, the percutaneous equivalents were 10 mg. and 80 mg., respectively; the peroral equivalents were well over 100 mg. for both substances.



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## ANIMAL PIGMENTS

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The prevailing conditions have resulted in few important papers appearing in this field during the period covered by this review. Many papers are inaccessible; others, dealing primarily with improvisations to meet local needs, require little discussion. Some, describing work with costly apparatus, show clearly that their authors have yet to acquire an adequate experience in the behaviour of the pigments investigated and in the interpretation of their results. Some authors do not even include enough experimental data to enable others to repeat the experiments described. I strongly deprecate the lack of energy which leads authors to publish the "percentage transmission" recorded by a photoelectric spectrophotometer instead of converting it to "extinction coefficient." This often renders virtually impossible an exact comparison of work so published with pre-existing data.

The time appears to be ripe for a competent committee to assign to the chief biological pigments names, constitutions, and the most probable values for their chief physical characteristics. The absorption spectra of haematin in alkali and of cyanhaematin, to take an example, recorded in the distinguished paper by Barron and his colleagues (1) differ greatly from those previously found (2, 3). An examination of the concentrations used shows that, in all probability, these substances obey Beer's law only at the extreme dilutions for the study of which the Americans' special equipment alone was suitable. This important point merits verification.

*Nomenclature.*—The question of nomenclature is as unsettled as ever. Lemberg (4) attributes this to the complexity of the subject; I cannot entirely agree. It is most regrettable that the admirable proposal, which emanated from both Europe (5) and the United States (6) to use "haemi-" as a prefix to denote compounds of a ferri-porphyrin, has been disregarded by the highest authorities in the latter. The contraction would have been suitable in French, German, and English; it could possibly have been adopted in Russia and in the languages of southern Europe. The mutilation, in the American tongue, of "haemo-" to "hemo-" leaves "hemi-" with two possible meanings. If "semi-" were always used for "half" I doubt if any con-

fusion would result. Were the old botanical practice, revived by Drabkin, adopted of denoting denaturation by the change of the terminal "-in" of a protein's name to "-an" and the cumbersome "-chromogen" reduced to "crom" and restricted, where possible, to classes of compounds, the nomenclature would be sufficiently logical, precise, and compact for practically all lectures, discussions, and papers. Thus "denatured globin ferrihaemochromogen" would become "haemigloban"; and "parahaematin," "ferrihaemochromogens," or "oxidized haemochromogens" would be "haemicroms." The economy of text alone should be tempting. The prefixes "proto-," "meso-," etc. could still be used where requisite. The Clark-Drabkin (7) terminology, like that of Fischer, could be reserved for special occasions. The vocal confusion possible between "haemi-" and "haemo-" could be avoided as is that between "ferri-" and "ferro-."

A worse and avoidable fault in nomenclature is the common one (8) of using "hemoglobin" to signify any and every derivative of the blood pigment which contains native globin and occasionally some whose constitution is most dubious. It is most doubtful if the protein of choleglobin is indeed globin. "Verdo-" in some quarters (9 to 13) is being used for any green derivative of haemin or haemoglobin regardless of its constitution. Lemberg (4) makes the distinction clearer.

#### THE DETERMINATION OF PIGMENTS

As usual a large number of the papers deal with the determination of various pigments under diverse conditions of admixture, speed, complexity of equipment, etc. Few fresh principles are advanced, many of the papers describing the adaptation of old methods to such modern refinements as photoelectric or spectrophotometric apparatus. Some of the divergent views expressed appear to be due to an inability to realise the varied requirements underlying an apparently single need. There are, too, considerations which act against each other: the use of costly labour-saving devices or of less elaborately trained personnel, the various sizes of samples permissible in special circumstances, and the different substances which it is desired to include or exclude under the title "haemoglobin."

Despite modern equipment biochemical determinations should still be based on the thoroughly tested fundamental principles of analysis. The following conditions are as essential as ever: (a) If it be in any way possible, a heterogeneous sample (e.g., a suspension) must be

converted to a homogeneous sample. (b) The form of the substance on which the final measurement is made must have a definite composition. (c) The purely metrical errors must be kept small compared with the permissible error in the whole analysis. (d) An apparatus or method must be calibrated by the analyst who uses it. (e) The most specific method deserves the most consideration.

How far a minute specimen may serve as a reliable sample has been studied by Brückmann (14) who wisely opposes the use of small ear-specimens of blood when a larger specimen can be procured from a finger or an arm-vein. In my opinion (see also 15) the usual 20 c.mm. commercial pipette contributes to some of the curious results obtained, owing to the difficulty of securing sufficient consistency in the amount of blood which it contains. I found a 50 c.mm. bulb pipette which I blew from a very fine capillary tube more consistent. Another unrecognized source of variable results is the condensation of moisture within the pipette when it is cleaned with ether and air is drawn through it. I strongly support those who advocate haemolysis of a blood specimen before it is pipetted. In many impressive methods the difficulty of measuring accurately a given sample of blood, without either sedimentation or entrainment of minute air-bubbles, is apt to be the limiting factor. In other methods it is the use of a wide graduated tube for measuring the final volume before the colorimeter or photometer is applied. The demand, for purposes of industrial hygiene, for greater accuracy and reliability in haemoglobin analyses warrants a critical survey of every aspect of the determination before costly equipment is installed. Even in the most elaborate methods for oxygen-capacity determinations only the content of oxygen is measured unless the blood has been carefully saturated with oxygen immediately prior to the determination. When this is done simpler apparatus, such as that used by Gibson (16), may serve.

There is an increasing tendency to limit the light used for colorimetry to narrower wave-bands and to provide quasi-permanent standards of reference, i.e., for the colorimeter to approach the spectrophotometer. There is also growing a most reasonable preference for the determination to be made on a derivative of haemoglobin of definite constitution rather than on a haematin with its uncertain degree of aggregation and, in consequence, of absorption (17). Some of the standards suggested, such as coloured gelatin films between glass plates, are in reality far from permanent. In view of the increasing availability of precise colour screens made entirely of glass, it

should not be long before a simple commercial colorimeter is on the market using green light and determining oxyhaemoglobin as such against a permanent glass standard. Such an instrument utilises chiefly the absorption due to the  $\beta$ -band of oxyhaemoglobin. The green screen prevents the red rays which are freely transmitted by many blood pigment derivatives from impairing the discriminatory power of the eye. Thus is avoided the chief disadvantage of methods based on the comparison in daylight of haemoglobin, carbon monoxide haemoglobin, or cyanmethaemoglobin with a permanent standard. The author (18) made up such an instrument employing glasses locally procurable and found it quite satisfactory. For a photoelectric version of this method see the article by Bell & Guthmann (19).

Donaldson (20), in a discussion of haemoglobin standards, mentions that a standard tube of carbon monoxide haemoglobin made by the late J. S. Haldane had varied very little in thirty years. The paper helps to explain some of the difficulties in colorimetry with white light but, as indicated above, the trend in analysis seems to be towards a selected monochromatic illuminant.

Bell & Guthmann (19) have published a good description of a fairly simple photoelectric haemoglobinometer in which the light intensity is varied by varying the distance between the lamp and the photo-cell. The blood pigment is determined as oxyhaemoglobin using apparently a colour screen somewhat similar to that described above (18). They, too, note the difficulty of consistent measurements on small volumes of blood.

Peterson & Strangeways (15) describe a photoelectric haemoglobinometer in which a variable graduated slit is used to adjust the light passing through an alkaline haematin solution till it equals that which has passed through a permanent standard. The instrument, of the two-cell null-reading type, uses one cell alternately for the standard and the unknown solution and the second cell to compensate for fluctuations in the light source. The details given of design, construction, and performance are insufficient for duplication or criticism. It remains to be seen whether these papers make any important contribution to the solution of the three major problems of photoelectric instruments: elimination of, or compensation for, variations in the source of light, fatigue of most photoelectric cells, precise photometry, or the achievement of a linear response to light intensity of the required degree of accuracy. When possible, experts will adopt, as the logical method of pigment analysis, the spectrophotometer, especially

of the photoelectric type. In no other way can they so effectively exploit the specific properties of the substance to be determined. There are, however, likely to be for a long time many workers for whom such an instrument is out of the question. For such the solution probably lies in the use of artificial light of a suitable colour with permanent glass standards of reference for comparison, personally calibrated. With due application they may yet have the consolation of knowing that their limitations are more of speed and convenience than of accuracy.

Roughton & Scholander (21, 22) describe an apparatus for the determination of carbon monoxide and of oxygen in 40 c.mm. of blood which consists of a glass syringe with a capillary gas burette fused to it. Rosenthal & Drabkin (23) determine cytochrome-*c* by precipitation with trichloroacetic acid after heat coagulation of interfering pigments in half-saturated ammonium sulphate solution. The cytochrome-*c* is redissolved and its concentration measured by spectrophotometry of its reduced form.

Rimington (24) describes the separation of porphyrins from other urinary pigments by adsorption on kieselguhr. Other work on the comparison or standardization of analytical methods is mentioned in the bibliography (25 to 30). The recent literature includes discussions of haematin methods (31, 32), fluorescence methods (33 to 36), and methods for the analysis of bile and allied pigments (37 to 44).

While on the subject of analysis, a few papers on the use of haemoglobin as an analytical reagent may be noted. Hill (45) used muscle haemoglobin as a reagent for the determination of small amounts of oxygen. Various suggestions have been made from time to time for the use of blood haemoglobin for the determination of small amounts of carbon monoxide in air. One recent method appears to rely too much on the maintenance of a set of standard conditions (46). It seems certain from the description that the carbon monoxide present would be by no means quantitatively absorbed. A second method, using only about 25 cc. of air for a determination, and a very simple apparatus, is so nearly quantitative in its reactions that it is theoretically preferable (47).

#### PHYSICAL DATA

Magnetic data for ferritin and haemosiderin are presented by Michaelis, Coryell & Granick (48). Granick (49) notes that crystal-

lized ferritin from testes contains little or no iron. One wonders about the significance of magnetic determinations on a material of so variable a content of iron.

Boyes-Watson & Perutz (50) extend the latter's work on the x-ray analysis of crystals of oxyhaemoglobin. They describe the dimensions of the molecule and its general structure. O'Daniel & Damascke (51) from x-ray studies assign a planar structure to the molecule of tetramethylhaematoporphyrin. Fankuchen (52) reports that the x-ray data for ferritin and apoferritin are the same; the absence of iron from the latter compound does not alter the shape of the packing of the protein molecules.

Bücher & Negelein (53) measured the number of quanta of light energy required to liberate one molecule of carbon monoxide from carbon monoxide haemoglobin. When dissolved in water four quanta are required for each molecule of carbon monoxide set free; when dissolved in 2.6 *M* sodium chloride two quanta are necessary. In the latter solvent the molecular weight is only half that in water. Carbon monoxide is liberated from its compound with myoglobin with the expenditure of one quantum per molecule. It is argued from these results that the quantum yield is related to the degree of interaction between the haem molecules in the pigment. Havemann (54), who has recently published a good deal of rather indecisive analytical work (55, 56), differs from Taylor & Hastings (57) on the symmetry of the oxidation-reduction potential curve of haemoglobin-methaemoglobin. Roberts (58) measured the heat of reaction of methaemoglobin and of carbon monoxide haemoglobin with sodium salicylate. With carbon monoxide haemoglobin he found a slow evolution of heat, incomplete after one hour, which, I think, might be associated with a slow oxidative denaturation since oxygen was probably not completely excluded. With methaemoglobin the heat was liberated more promptly and increased steadily with the amount of sodium salicylate used up to 0.8 molar. Roberts concludes that it is improbable that the reaction is stoichiometric; he believes that part of the heat is due to hydrogen bond formation between the salicylate ion and side-chains of the protein. His spectrophotometry is less convincing than his thermochemistry, though he confirms Anson & Mirsky's (8) work on the effect of sodium salicylate on the spectrum of alkaline methaemoglobin. Particularly hard to understand is his statement that the spectrum of alkaline methaemoglobin in the presence of sodium salicylate resembles that of carbon monoxide haemoglobin in its absence. It is inter-

esting to notice that sodium benzoate produces a similar change of spectrum without any evolution of heat.

In an extensive research Barnard (59) has made a number of physical measurements on haemoglobin, methaemoglobin, and oxyhaemoglobin. The measurements include pH titrations and determinations of the conductivity and specific inductive capacity of solutions of these derivatives under varying conditions of pH and salt content. His main conclusions seem to be: (a) Haemoglobin and methaemoglobin have identical degrees of base receptivity. (b) The acidity, basicity, and buffer powers of haemoglobin are increased by the addition of sodium chloride or bromide. (c) The Debye-Hückel theorem is invalid for dipolar ions.

Two mathematical investigations should receive mention here. The first is by Legge (60) who, from an examination of the data of Brooks (61) on the autoxidation of oxyhaemoglobin to methaemoglobin, suggests that an intermediate compound,  $\text{Hb}_4\text{O}_2$ , is transformed spontaneously to methaemoglobin. The second is by Eley (62) who critically analyzed the data of Hartridge & Roughton (63, 64) on the rates of formation of oxyhaemoglobin and of carbon monoxide haemoglobin. As the result of his analysis he concludes: (a) The reaction of haemoglobin with carbon monoxide appears to be relatively normal by the standards of chemical kinetics. (b) That with oxygen is abnormal;

the association reactions are marked by their low value of the activation energy, but the dissociation of oxyhaemoglobin by the high value of the entropy of activation. It is exceedingly unlikely that this is an unreal value due to a chain reaction. It is most probably due to a loosening of the internal structure of the protein on forming the activated complex.

Whatever an extension of this type of approach may mean in the future it seems to urge one consideration now. As I have long felt, the similarity in spectral type, reinforced by magnetic data, coupled with the ease of transition from one to another has, in the minds of many, conferred on these two derivatives of haemoglobin a spurious similarity. It may well be that in carbon monoxide haemoglobin the carbon monoxide, as in its compound with haem, is directly joined to the iron atom of the haem molecule; in oxyhaemoglobin on the contrary the oxygen molecules may well be attached to other portions of the protein and a reshuffle of the linkages throughout the protein occur. This could confer on the prosthetic group the appropriate elec-



tronic configuration that is associated with diamagnetism and the two-banded visible spectrum.

*Spectra of pigments.*—A few data on the spectra of pigments have appeared. Kiese & Kaeske (65) give a list of the wave lengths of the visible bands of various derivatives of haemoglobin, myoglobin, and verdohaematin. Bechtold (66) reports various changes in the spectrum of myoglobin after treatment with sodium nitrite. Horecker (67) has measured the infrared spectra of several haemoglobin derivatives. The most interesting aspects of this work are the difference between oxyhaemoglobin and carbon monoxide haemoglobin, the latter having no infrared band—alkaline methaemoglobin has a band at 8000 Å. Loyarte *et al.* (68) have published determinations of the ultra-violet absorption spectra of human and guinea pig blood, with and without carbon monoxide. Roy (69) found that bilirubin obeys Beer's law when dissolved in serum but not in water at the same pH.

Jope *et al.* (70) determined the iron, oxygen capacity, and molecular absorption coefficient ( $E$ ) of the Soret band of twenty-five specimens of human blood. The oxygen capacities varied from 367 to 418 cc. per gram of iron (theory 401). The  $E$  of the Soret band varied from 127 to  $141 \times 10^3$  with a mean of  $132 \times 10^3$ . A specimen of dried horse oxyhaemoglobin, according to their work, had an oxygen capacity of 409 which seems remarkably good.

Barnard (71) has published a paper on the effect of saccharin in very high concentration on the spectrum of an unspecified methaemoglobin, with which he considers it to combine. At pH 6.0 the protein was precipitated, but remained soluble at pH 7.4. The spectral change observed is reminiscent of those already described (8) when sodium salicylate is added to alkaline methaemoglobin. No details are given of any other properties of this new compound the existence of which seems to rest on slight evidence. No evidence for the denaturation is offered apart from the precipitation and at pH 6.0 precipitation and denaturation do not necessarily go hand in hand. Since no details are given of the source or method of preparation of the methaemoglobin the work cannot be repeated with any certainty.

McCarthy (72, 73) has published two papers on foetal haemoglobin. He finds that it has the same osmotic pressure as maternal haemoglobin but a lower affinity for oxygen when in aqueous solution. In the maternal corpuscles, however, the haemoglobin's affinity for oxygen is markedly diminished by other substances present, while in the foetal corpuscle this is not the case (cf. Baar & Hickmans, 74).

Ponder (75) attributes the variable relation between corpuscular density and iron content to the presence of an iron-free porphyrin globin. Apart from the lack of supporting evidence it would appear that variations in the amount of stroma might account for the divergences. According to Ginetsinskii (76) the affinity of haemoglobin for oxygen falls with increasing concentration of haemoglobin. This was not noticed by Forbes & Roughton (77). From their papers some idea may be gained of the difficulty of the problem, particularly as regards the alterations undergone by the pigment at low concentrations. McCarthy (78) has found a physiological diurnal variation in men of as much as 7 per cent in the carbon monoxide capacity of the blood. If this is to be referred to changes in the concentration of the blood pigment it is surprising that more is not heard of it.

#### DISINTEGRATION OF THE BLOOD PIGMENT

One of the more mobile fields of pigment research is that which includes the territory bounded on the one side by haemoglobins and the protein-haem enzymes and on the other by the recognized products of the disintegration of haematin, e.g., biliverdin and the bilins. It seems fairly certain that most of the substances described between these groups are mixtures of compounds of diverse constitutions. Thus it is now known that sulphhaemoglobin formed *in vitro*, unlike clinical sulphhaemoglobin, is heavily contaminated with choleglobin-like compounds (79). Pseudohaemoglobin and its like have apparently a good deal of cyanmethaemoglobin or cyanhaematin mixed with them. Bingold's (12) observations on the effect of hydrogen peroxide on heated oxyhaemoglobin deserve further study though he appears to make the common mistake of inferring that a green colour is proof of the "verdo-" structure. Haurowitz's (80) sulphur-containing porphyrin likewise deserves a most thorough investigation.

Recently the author (81) has prepared from horse oxyhaemoglobin a green pigment "cruoralbin" by the concurrent action of potassium cyanide, ammonium chloride (as a buffer), sodium hyposulphite, and oxygen. It appears to contain little unchanged protohaematin pigments and the losses due to denaturation are much less than when ascorbic acid and oxygen are used. The absorption band in the red of its carbon monoxide compound has a higher coefficient than that calculated by Lemberg for carbon monoxide cholehaemochromogen (82). The protein, though still native, is apparently altered during

the reaction and is no longer the globin of haemoglobin. Its relation to the other substances of this group is unknown.

#### CHEMICAL STUDIES

Lemberg (4) has closed the ring of verdohaematin with ammonia, forming monoazahaemin. He has similarly prepared monomesoaza-haemin. Also, he has recorded the spectra of some derivatives of these compounds. This provides a clear distinction between the "chole-" and "verdo-" series of derivatives. Stier (83, 84), using Lemberg's technique, has split the rings of deuterohaemin and of some other haemins. He considers that verdoparahaematin is a mixture of at least two oxidation stages. Stier & Gangl (85) with colloidal palladium in pure formic acid reduced coproverdohaemin ester to a mixture containing coproporphyrin I tetramethyl ester and coproglau-cobilin ester. Lichtenwald (86) unsuccessfully attempted a synthesis of vinylneoxanthobilirubinic acid from the appropriate pyrromethene. He and Plieninger (87) describe the syntheses of a number of pyrromethenes by the condensation of hydroxypyrroles with pyrrole- $\alpha$ -aldehydes. Grinstein & Watson (88) have published improved methods for the purification of protoporphyrin by crystallization and of its methyl ester by chromatography. Polonovsky's papers (89) on the effect of ethyl hydroperoxide on methaemoglobin are unfortunately not available to me. Theorell (90) adds his weight to the condemnation of Bechtold's (91) unfortunate claim to have converted myoglobin to cytochrome-*c*. The latter's defence (92) of his position serves to underline the untrustworthiness of unsupported spectroscopic data.

Keilin & Hartree (93) extend Jung's (94) observations on nitrosobenzene haemoglobin and describe nitrosobenzene haem. Siedel & Winkler (95) have synthesized etioporphyrin and other porphyrins by methods based on oxidation of pyrrole derivatives with lead tetraacetate. Lemberg & Legge (96) have shown that the bile-pigment haematin in liver catalase is produced during its action on hydrogen peroxide. Schultze (97) has isolated protoporphyrin IX from the faeces of both normal and anaemic rats. Jope & O'Brien (98) have contributed observations on the irregularity of the human excretion of coproporphyrin.

#### THE STRUCTURE OF THE BLOOD PIGMENT

Current views on the structure of haemoglobin derivatives are based chiefly on titration and oxidation-reduction curves, magnetic and spectroscopic data. It is tacitly assumed that the various pros-

thetic groups are attached to the same portions of the protein molecule and that a substance investigated is an individual.

Though the titration curves, magnetic and spectrophotometric data of the various American scientists have presumably established the immediate relationships of the prosthetic group to the protein of the blood pigment there are a few facts which their scheme does not appear to cover quite satisfactorily. Russell & Pauling (99) found that the imide group of the iminazole molecule which is covalently linked to the iron atom in iminazole methaemoglobin had a  $pK'$  of 9.5. Theorell (100) ascribed two acid groups— $pK'$  9.3 and 9.85—in cytochrome-*c* to the imide groups in the histidine residues similarly covalently linked. In methaemoglobin itself it is said (101) that similarly situated imide groups of the histidine residues have  $pK$ 's 5.25, 6.65, 8.1 while the corresponding values for carbon monoxide or oxyhaemoglobin are 5.75, 6.80 and for haemoglobin 5.25, 7.81. Apparently the  $pK'$  of an iminazole imide group possesses a facile variability with changing circumstances. If other groups are as accommodating, the assignation, in a complex and incompletely analyzed protein, of a given  $pK'$  to a particular acidic group is perhaps not final. Again, the apparent heats of ionisation of the groups associated with the iron atom, while approximating that of iminazole in the case of the blood pigment derivatives better than do the apparent dissociation constants, are too high in the case of cytochrome-*c* where the agreement as regards the dissociation constants seems to be better. As far as the evidence quoted above shows anything it suggests that each of the blood pigment derivatives possesses two acid groups whose dissociation constants vary comparatively little with the valency of the iron atom or its change from the ionic to the covalent state. The acidities of these groups, however, differ considerably from that of the iminazole molecules coordinately linked to an iron atom in iminazole methaemoglobin.

Ultraviolet spectrophotometry of porphyringlobins has shown that such substances may have an intense Soret band (102). The presence of this band in haemoglobin or methaemoglobin is thus no evidence that the pigment is attached to the globin by the iron atom. The magnetic susceptibilities of methaemoglobin, cyanomethaemoglobin, fluoromethaemoglobin, haemoglobin and, probably, carbon monoxide haemoglobin are the same as those of the corresponding protein-free pigments. The differences in the visible spectra between the analogous members of these two groups are small.

The spectra of the compounds of globin with protoporphyrin and with its copper, zinc, and cobaltous derivatives bear to those of the free pigments a relation very similar to that existing between, say, methaemoglobin and acid haematin, or carbon monoxide haemoglobin and carbon monoxide haem. It is less easy to assign to these compounds structures as analogous to those of haem derivatives as their observed spectroscopic properties appear to warrant. A very interesting paper by Keilin (103) describes changes in the visible spectra of certain derivatives of haematin and of protoporphyrin which are brought about by the addition of caffeine. These changes resemble those due to the presence of a protein. Caffeine does not form a haemochromogen but appears able to aid somehow the dispersion of the pigment molecules. It would be difficult to deduce from these facts that in these cases the iron atom of the prosthetic group is involved in its union with the protein. If protoporphyrin globin is any guide, one might suspect, rather, the carboxyl groups of the prosthetic group to be responsible. Alkaline methaemoglobin, which has a magnetic susceptibility intermediate between that of methaemoglobin and a fully co-ordinated compound, such as cyano-haematin, may well be a mixture of a co-ordinate compound of globin with haematin, analogous to denatured globin para-haematin, and methaemoglobin. Since increasing the hydroxyl ion concentration beyond about pH 11 dissociates alkaline methaemoglobin to "alkaline haematin," a more paramagnetic substance, there will be a pH zone of minimum susceptibility, simulating the formation of a definite compound with a peculiar susceptibility. Neither its visible spectrum, which resembles that of denatured globin para-haematin, nor its ultraviolet spectrum, with its small distorted Soret band, controverts this view. Casein and denatured hen ovalbumin form para-haematins in alkaline solution which are dissociated at the neutral point. Their behaviour is in sharp contrast to that of denatured globin para-haematin, but serves to emphasize the limitations of current knowledge.

The amino-acid analysis of globin is still very incomplete; the basic haemochromogenic groups may be more important in connection with its iso-electric point than with its unique property of forming haemoglobin for which the unknown portions may be more significant. On this subject the chemistry of the blood pigment has thrown little light. Now that a start has been made on the task of performing chemical manipulations without denaturing the globin, more information about the peculiar structure of the globin molecule may be hoped for.

The relations between hydrogen cyanide and the blood pigment provide a set of curious phenomena deserving attention. Its action in converting oxyhaemoglobin to methaemoglobin seems to depend on the presence of feeble reducing agents—ascorbic acid accelerates it. Recrystallized oxyhaemoglobin is but slowly converted. Since hydrogen peroxide alone has little effect on oxyhaemoglobin it is difficult to understand this reaction or the joint reaction of peroxide and cyanide on oxyhaemoglobin unless it is assumed that the effect of the cyanide is to convert a small portion of the blood pigment into a derivative readily attacked by the peroxide. Were such a portion converted into (native) globin haemochromogen the reactions would, at least, align with the preparations of verdohaemochromogen from haematin using peroxide, pyridine, and hyposulphite or hydrazine and oxygen. The preparation of cruoralbin would also fall into line. That the diamagnetic substance first formed by the reduction of cyanmethaemoglobin is cyanothaemoglobin has not been independently established. Its ready disintegration by oxygen is decisively against this view. Its visible spectrum resembles that of a haemochromogen which would also be diamagnetic. It has never been prepared from hydrogen cyanide and haemoglobin and, in my experience, even under the most favourable conditions slowly reverts to haemoglobin.

Another unilluminated aspect of the haem-cyanide relationship is the well-known fact that with cyanide haem forms three compounds, spectroscopically distinct. With very low concentrations of cyanide at about pH 8 the visible spectrum is that of a typical haemochromogen with its intense  $\alpha$ -band. With increasing concentration a second spectrum of several vague bands appears. The third, the well-known cyanhaemochromogen, appears with higher concentrations of cyanide and more alkaline solutions. That the second is not a mixture of the first and third together with some uncombined haem is indicated by the fact that, in my experience, with acetonitrile in place of cyanide the first two spectra can be obtained but not the third. It has been asserted that the first is a compound of one molecule of hydrogen cyanide with one of haem. Its close spectral resemblance to pyridine haemochromogen seems, however, to demand for it the same constitution as the latter, namely, two molecules of nitrogenous substance to one molecule of haem. The dissociation of haemochromogens in solutions dilute enough for spectrophotometry, even with special cells, renders difficult the exact analysis of these compounds.

Thus the known behaviour of haemoglobin derivatives is consonant

with the idea that the normal link between protein and prosthetic groups is by suitable basic groups to the carboxyl groups of the latter. In haemoglobin a very minute amount, in alkaline methaemoglobin more, and in oxyhaemoglobin all of the prosthetic iron is covalently linked to the protein, perhaps in addition to the carboxyl linkages. Hydrogen cyanide would appear not to be covalently linked to the ferrous atoms of haemoglobin but to favour the covalent linking of the ferrous atoms to the globin.

#### VISUAL PIGMENTS

The following groups of papers have appeared on the subject of visual purple. Broda & Goodeve (104) have compared its spectral changes on cooling to  $-73^{\circ}\text{C}$  with those of other substances under these circumstances. Busch (105) found that a little Zephirol clarifies a solution of visual purple but that much denatures it. Peskin (106) found that the only effect of previous exposure to intense light of an intact eye of a frog was to delay for ten minutes the start of the subsequent dark adaptation. Chase & Hagan (107) state that solutions of frogs' retinæ containing visual purple show the same changes in absorption spectra when illuminated by white light whether oxygen be present or excluded. Krause (108) extracted a yellow lipid from ox retinæ. On alkaline hydrolysis it is broken down to a fat and a substance which is not visual yellow and which he provisionally names "indicator yellow."

#### OTHER PIGMENTS

A number of papers on other pigments are included in the bibliography but cannot be discussed in detail. They include studies on the pigments of various organisms and are mostly descriptive. A few may be mentioned here. Koschara (109) describes the isolation from urine and the synthesis of xanthopterin. Rothman & Flesch (110) have isolated a red iron-containing pigment from human red hair. Brohult (111) has measured the molecular weight of haemocyanin of *Helix pomatia*. Rawlinson (112) studied the effect of oxidizing agents on the haemocyanin of *Jasus lalandii*. Working with scrupulously purified material he was unable to find conclusive evidence that this haemocyanin can be oxidized to a methaemocyanin by any of the most powerful oxidizing agents available, thereby differing from the haemocyanin of *Limulus polyphemus* studied by Conant (113). He observed colour and other changes similar to those reported by the American

authors but after a careful investigation came to the conclusion that in his case they were due to extraneous effects connected with the action on the protein of the oxidants used. The potential changes observed during the oxidation have been ascribed by him to similar adventitious causes. The matter has some bearing on the theory of copper-containing respiratory pigments and seems to warrant further critical study.

Bliss (114) has shown that the red pigment of the retina of the squid and of some varieties of crab, is converted by treatment with formaldehyde to photosensitive substances which are bleached by light. This appears to be a new type of reaction and to offer most interesting possibilities for future work.

Other recent papers on animal pigments which have not been discussed in the text include the following, many of which are essentially descriptive in character. The molecular weight and iso-electric point of the erythrocrucorin of the marine lamprey (115); acridioxanthin, a brown pigment of grasshoppers (116); a comparison of the pigments of Peruvian cloth with those of *Coccus cacti* (117); green pigments of fishes (118); protoporphyrin in the shells of birds' eggs (119); an ether-soluble pigment from the retinæ of ring-vipers freed from oil granules and visual purple (120); eye, skin, and wing pigments of insects (121); the appearance of brown pigments in egg-shells shortly before ovideposition (122); a yellow pigment of lizard skin (123); a rose-coloured pigment of Lampyridæ (124); the pigments of the erythrocytes of the sea-urchin (125); the synthesis and structure of echinochrome (126);  $\alpha$ -cytochrome in the muscles of the cod and the green herring (127); ichthyopterin, a fluorescent pigment of fish skin (128); hepatoflavin in the livers of different animals (129); a pigment of the bronchial hearts of the octopus (130).

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## DETOXICATION MECHANISMS

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The present review is a discussion of selected topics which have appeared in the literature from 1940 to October, 1944. By way of introduction little need be added to the general remarks prefacing the last review on this subject (1). The point of view taken by Stekol toward this subject has been substantiated and amplified during the period covered by this review. While the disappearance of many drugs after administration is of great interest to pharmacologists, the present review will be limited to those studies in which the nature of the reaction involved has been defined.

The teleological implications inherent in the term "detoxication" have been seriously discredited during recent years. Whereas the term is used throughout this paper, it does not necessarily imply that a nontoxic or less toxic material is produced. Only a limited amount of work has been done in comparing the toxicity of the parent compound with its detoxified product. However, a few illustrations will suffice. While the sulfonamides are all more or less toxic, their acetylated derivatives are capable of damaging the renal tubules because of their insolubility (2). Relatively innocuous compounds such as nicotinamide (3) and glycocyamine (4), both normal metabolites, when fed in sufficient quantity produce methionine and choline deficiency, as a result of their methylation. Acetoin, which may be a product of alcohol metabolism, is appreciably more toxic than the original substance and results in the same symptomatology (5). Peculiarly, while some detoxications proceed with great speed and commence within a few minutes after administration of the substance in question (6, 7), when single lethal doses of camphor or phenol are given the rate of detoxication is seriously delayed (8).

Rather than postulate the existence of a large number of enzymes capable of rendering the host of synthetic products of organic chemistry less toxic to the animal organism, it now seems more reasonable to suggest that foreign substances may only undergo chemical transformations physiologically when they possess a structural group similar to some normally occurring metabolite. This chemical group-

ing then fits into the enzyme system which handles the normal metabolite and "detoxication" is accomplished. It is the complexity of normal metabolism which permits the variety of reactions to be reported herein.

Studies of detoxication are nonetheless valuable. They provide information which is desirable from several points of view. Primarily they describe the enzyme systems present within the animal and thereby elucidate normal metabolism. The simultaneous presence within a foreign molecule of a grouping which is attacked by some enzyme system and of some other group which remains unchanged permits the isolation of products which establish the nature of the reaction. This is particularly evident in the history of the study of fatty acid oxidation. By studies of the fate of drugs, valuable information is obtained for the synthesis of new and superior therapeutic agents. And finally such studies should provide the working basis for satisfactory liver and kidney function tests.

*Acetylation.*—Current interest in *p*-aminobenzoic acid and the sulfonamides has stimulated investigations on the mechanism of acetylation. Fishman & Cohn (9) found that when the body water of rats was maintained at a level of 2 to 3 per cent deuterium oxide, and either *p*-aminobenzoic acid, sulfanilamide or *d*-phenylaminobutyrate were fed, the acetylated derivatives which appeared in the urine contained deuterium in the acetyl group only. After feeding sulfanilamide with acetic acid containing deuterium in the methyl group Bernhard (10) found that 12 per cent of the acetylsulfanilamide of the urine of rabbits and 9 per cent in the case of men had been formed from the dietary acetic acid. Essentially identical results were obtained with *p*-aminobenzoic acid. When deuterioethanol was fed with sulfanilamide (11) as much as 24 per cent of the acetylated sulfanilamide appeared to have come from the dietary ethyl alcohol. Bernhard & Steinhauser (12) also fed *l*- and *d*-cyclohexylalanine with deuterium labeled ethanol and acetic acid and found only acetyl-*l*-cyclohexylalanine in the urine, and, similarly, in all cases the acetyl group appeared to have arisen from the dietary alcohol or acetate. They concluded that acetate may be directly used for acetylation. In contrast to previous reports they found no acetyl derivatives after administration of phenylaminobutyric acid to rats or dogs. Bernhard has also fed sulfanilamide with deuteriosuccinic acid and found no excess deuterium in the acetylsulfanilamide of the urine (13). However, because of the great biological lability of the hydrogen atoms of the

succinate molecule, negative results were not conclusive, although positive results would have been.

In accord with these observations are the findings of Klein & Harris (14) that acetate increased the acetylation of sulfanilamide by rabbit liver slices and of James (15) who found an increased excretion of acetyl sulfanilamide when acetate was given with sulfanilamide. He also confirmed the previous findings (16) that the acute toxicity of acetyl sulfanilamide was greater than that of sulfanilamide. James (17) also observed a drop in the alkali reserve of rats, after giving sulfanilamide, which was prevented by giving acetate. His explanation in terms of the withdrawal of acetate precursors by the sulfanilamide is probably incorrect and the phenomenon is more probably related to the inhibition of carbonic anhydrase (18). In contrast are the results of Doisy & Westerfeld (19) who found no increase in acetyl *p*-aminobenzoic acid excretion after acetate administration to rabbits but who observed a marked increase when acetoin or 2,3-butylene glycol were given. Paraldehyde administered to mice was found to increase the excretion of acetyl sulfanilamide (52). Martin & Rennebaum (20) found an actual decrease of 40 per cent in the formation of acetyl sulfanilamide, as judged from blood levels in rats, when acetate was given with the sulfanilamide, while pyruvate administration actually increased it by 300 per cent. Oddly enough, they observed no effect when lactate was given, yet did find increments after giving glycine, succinate, cysteine, ascorbic acid, and acetoacetate. Acetyl sulfanilamide formation was decreased in thiamine and riboflavin deficiency. They state that insulin and epinephrine did not increase acetylation, while their data seem to show definite decreases after administration of these two drugs. However, considering the wide variations reported in the control animals given sulfanilamide alone and the lack of data concerning water intake and kidney function in these animals, more confidence might be felt in the results of these authors if they had, instead, measured total acetyl sulfanilamide excretion. The authors assert that these data substantiate the hypothesis of Lipmann (21) that acetyl phosphate, arising from pyruvate by way of phosphopyruvate, serves as the acetylating agent. Unless acetate may be directly converted to acetyl phosphate, it is difficult to reconcile this assertion with the findings of Bernhard (10, 11, 12) and of Bloch & Rittenberg (22). The latter authors have confirmed the findings of Bernhard with deuterium labeled acetic acid and attempted to determine the source of the acetate in normal metabolism. Deuterium con-

taining test substances and phenylaminobutyric acid were added to the stock diet of rats and the isotopic concentration of the excreted acetyl derivative was determined. Deuterioacetyl groups were excreted after giving ethanol, butyric acid, alanine, *n*-valeric acid, and myristic acid, but not from propionic acid nor 10,11-dideuterioundecylic acid. A considerable fraction of butyric acid appeared to yield 2 moles of acetic acid. Those compounds which gave rise to deuterioacetyl groups also formed tissue deuteriocholesterol, in accord with the findings that acetic acid is a specific cholesterol precursor (23). However, the question of acetate production from carbohydrate precursors remained unresolved.

Of interest in this connection is the modification of the hypothesis of Stedman & Stedman (24) proposed by Baer (25) who, on the basis of an *in vitro* analogy, postulated that choline is first condensed with the keto form of pyruvic acid and that acetylcholine is then formed after oxidative decarboxylation.

Studies by du Vigneaud and collaborators on the importance of acetylation in the physiological conversion of *d*- to *l*-amino acids have been continued (26, 27). When phenyl-*d*- and benzyl-*d*-cysteine were fed to rabbits the urine was found to contain almost entirely the N-acetyl-*l*-derivatives. When the *p*-bromophenyl and *p*-bromobenzyl analogues were fed there appeared considerable quantities of acetyl-*d*-derivatives. No inversion occurred when the *d*-mercapturic acids were fed. When *o*-bromobenzyl-*d*-cysteine was fed only the *dl*-form of its acetyl compound could be isolated from the urine. In contrast when benzyl- and *p*-bromobenzyl-*d*-homocysteine were fed, direct acetylation appeared to occur to only a slight extent. No *d*-mercapturic acids were found in the urine after the administration of bromobenzene, benzyl chloride, or *p*-bromobenzyl chloride. The finding of acetyl *d*-forms in the urine has confused the picture somewhat, so that the question of acetylation as an integral step in this inversion mechanism remains as yet unsettled. In discussing this problem Stekol (152), who fed S-benzyl-*d*-cysteine to rats and man and found acetyl-*l*- and acetyl-*dl*-benzylcysteine as well as considerable quantities of unchanged S-benzyl-*d*-cysteine in the urine, concluded that the acetylation of both *d*- and *l*-amino acids proceeds directly and that, therefore, acetylation is not an essential step in the inversion of *d*-amino acids.

The rate of acetylation has been studied by many investigators (6, 7). No diminution in the ability of patients with myasthenia gravis

to acetylate *p*-aminobenzoic acid was found by Torda & Wolff (28) who conclude that this probably also means that the ability to form acetylcholine is not impaired in this state. Several species differences have been noted in the acetylation of the sulfonamides. While this process readily occurs in man, rabbit, mouse, rat, fish, and chick, it does not occur in dog or frog (29). But three species of toads were found to acetylate various sulfonamides and *p*-aminobenzoic acid; while the turtle (*Pseudemys elegans*) acetylates sulfathiazole only (30). From the data of Van Winkle & Cutting it appears that in the cat and rabbit both liver and spleen are sites of acetylation of sulfanilamide and of sulfapyridine (31). The extent of acetylation of the sulfonamides and the solubility of their acetyl derivatives have been studied by many workers, and only a few references can be given here as a bibliographic aid (32 to 42), to which should be added the excellent annotated bibliographies published periodically by Merck and Company. It is apparent that the acetylation of these various drugs proceeds at different rates in different individuals. For therapeutic purposes it would be desirable to find a bacteriostatically active sulfonamide which is at least relatively poorly acetylated, since the acetyl derivatives possess little or no antibacterial action. However, on this basis there is relatively little to choose among the various sulfonamides now available. The acetylsulfonamides seem to have been definitely incriminated in the urolithiasis and hematuria occurring in sulfonamide therapy. From this approach, sulfadiazine seems to be most advantageous, since it rarely produces urinary concretions (42). This may be related to the observations of Kohn (43) that the urine after sulfadiazine feeding contains an acetylated compound which does not give color with a thiobarbituric acid reagent. Of particular current interest was the finding of Harned & Cole (44) that quinine and atabrine lowered the ratio of free to conjugated sulfapyridine in rat urine.

No evidence of deacetylation of acetylsulfonamides has been detected (45). However, Abbott (46) found that the formyl, acetyl, and propionyl derivatives of glycine all yield extra hippuric acid when fed with benzoic acid to rabbits. This is in accord with the known availability of several acetyl-*L*-amino acids for rat growth (47). The hydrolysis of heroin by horse serum (48), of atropine and monoacetylmorphine by rabbit serum (49), and of mono- and diacetylmorphine by human, rabbit, and rat liver, kidney and brain (50) has been reported. While guinea pig liver readily hydrolyzes homatropine and atropine, rat, cat, and dog livers do not do so nor do the brains, kidneys, or sera



of these animals (235). The liver and serum of some rabbits also hydrolyze these drugs and the genetic relationships of this situation have been described (49). Oberst (51) presented evidence that heroin is completely hydrolyzed to morphine in the human body.

*Hippuric acid.*—The most frequently used liver function test involving a detoxication reaction still appears to be the hippuric acid test (52). That this substance is very readily excreted by normal kidneys has been shown by Schwei & Quick (53). Intravenous administration of the benzoate now seems to be preferable (54). Several investigators have stressed the need for sufficiently large urine volumes during the determination (55, 56) while proper adjustment of urinary pH (57) and the addition of 5 gm. of ammonium sulfate to each 10 ml. of urine have been found to increase the yield of hippuric acid (58); a direct correlation between body weight and hippuric acid excretion was also noted. In a study of the acute toxicity of benzoic acid (59) the 50 per cent lethal dose ( $LD_{50}$ ) was found to be about 1.7 gm. per kg. for rats and rabbits. Comparable data are not available for hippuric acid. Benzoic acid was found to inhibit the *d*-amino acid oxidase at  $10^{-4}$  M (60). Hippuric acid was only 8 per cent as active at equivalent concentrations. The synthesis of hippuric acid from benzoate and glycine *in vitro* (61) has been again confirmed (62) with the additional observation that concentrated liver pulp is as effective as liver slices. After feeding glycine containing  $N^{15}$ , Waelsch and Rittenberg observed that the concentration of  $N^{15}$  in the urinary glycine was three times as great as that in the glycine of glutathione from the same animals and concluded that glutathione is not the immediate source of glycine for hippuric acid synthesis (63). Leuthardt (64, 65, 66) found that guinea pig kidney synthesized hippuric acid as readily from benzoate and glutamine as with glycine, although rat kidney did not do so. Glutamine was found to be more effective than serine in promoting hippuric acid synthesis by guinea pig liver although rat livers were ineffective in either case.

Sodium benzoate markedly inhibits rat growth when added to low protein diets. Normal growth can be restored by further addition of glycine or sarcosine (67). The latter observation is in accord with the known demethylation of sarcosine to glycine (68, 69, 70). From analyses of pooled urines and carcasses of mice after benzoate feeding Polonovski & Boy (71) concluded that creatine synthesis was unimpaired while, in contrast, Terroine & Boy (72) observed a reduction of the creatinuria produced in rats by thyroid extract when benzoate

was added to a low protein diet. Substitutions in the *m*-position of hippuric acid accelerate the action of the hippuricase present in takadiastase preparations while *o*-substitution inhibits and *p*-substitution is without effect (73).

A review of several aspects of the metabolism of hippuric acid and related substances has appeared (74). When rations such as milk-egg, milk-meat, or even diets containing synthetic amino acids as the sole source of nitrogen are fed to dogs there is still considerable excretion of benzoic acid in the urine, presumably as hippuric acid and the glucuronide (75). The "endogenous" origin of these aromatic nuclei has not been definitely established. Phenaceturic acid was isolated from the urine of rabbits on a milk-egg diet (75). Of interest also is the finding that the  $\beta$ -phenylbutyric acid isolated from the glucuronide fraction after feeding *dl*- $\beta$ -phenylbutyric acid showed a rotation of  $+5.6^\circ$  while that isolated from the glycine conjugate fraction showed a rotation of  $-11.1^\circ$ . When *dl*- $\beta$ -phenylbutyrylglycine was fed the same fractions showed rotations of  $+9.2^\circ$  and  $-2.4^\circ$  respectively (75). This phenomenon warrants further study.

In contrast to many other reports, it has now been observed that in acute or chronic carbon tetrachloride poisoning, rats appear to be able to synthesize and to excrete hippuric acid (76).

The early observations of Ackermann (77) concerning the conjugation of nicotinic acid by the dog to form nicotinuric acid was confirmed (78) and was also found to be true in the case of man (79, 80) but not of rabbit (81) and rat (82). Neither man nor dog possess the ability to split nicotinuric acid when it is administered intravenously (78, 80). In the rat nicotinuric acid is split to nicotinic acid (82).

*Glucuronides.*—Little information has been gained concerning the metabolic origin of glucuronic acid. A diminished liver glycogen content has been found to result from the feeding of menthol or tertiary butylacetic acid (83), but this does not necessarily indicate direct utilization of glycogen for glucuronic acid production. The *in vitro* studies of Lipschitz & Bueding (84) have suggested that the 6-carbon chain is synthesized *de novo* from 3-carbon precursors.

There have been some improvements in methods for the estimation of uronic acids. A modification of the method of Maughan *et al.* (85) permitting the estimation of small amounts of glucuronic acid in bacteriological media was described by Ratish & Bullowa (86) and a photometric method for use with the step photometer was described by Florkin & Crismer (87). A most carefully worked out modification

of the naphthoresorcinol method as applied to urine has been presented (88), and by the use of this method it was found that three normal adults excreted 0.76 to 1.33 gm., average 1.05 gm., of glucuronic acid per day, which is considerably higher than previously reported values. A new procedure for the preparation of naphthoresorcinol for uronic acid estimations has become available (89).

The glucuronide of benzoic acid, which predominates in dog urine after dosage with sodium benzoate (90) has also been demonstrated in human urine (91) as has the glucuronide of phenylacetic acid (92). Borneol detoxication by man has been extensively studied. After oral doses of one or two grams of borneol thirty-four normal subjects excreted between 60 and 100 per cent as the glucuronide within twenty-four hours. The majority of a group of patients with mild or severe liver damage, as well as seven out of eight patients with nonhepatic disorders, excreted small amounts of borneol glucuronide under the same conditions (93, 94). In contrast, is the finding that borneol-glucuronic acid conjugation by rats was not impaired even when marked cirrhosis and malignant degeneration were produced by butter yellow (95). The capacity of patients with gastrointestinal cancer and secondary hepatic dysfunction to form glucuronides was seriously diminished although the production of phenol sulfate was found to be normal (96). Of interest in this connection is the observation that adrenalectomized cats do not conjugate phenols with glucuronic acid, while the production of ethereal sulfates is unimpaired (97).

The administration of such unrelated substances as dihydroxyacetone, glycerol, lactic acid, glycerophosphate, succinate, malate, saccharate, adenylate, cysteine, tyrosine, and sulfathiazole, all have been reported to increase glucuronide excretion (98). Pyruvate, acetate, glycolate, tartrate, formate, and citrate either produced small decrements in glucuronide excretion or showed no effect. Decreased excretion by rats was observed in deficiencies of pantothenic acid, thiamine, and pyridoxine as well as of vitamins A, E, and D (98). In contrast, riboflavin deficiency resulted in an increased production of glucuronides. The significance of the finding that glucuronic acid administration blocks the acetylation of sulfanilamide in the rat (99) is not clear, since the formation and excretion of a glucuronide of sulfanilamide was not demonstrated.

The appearance in the urine of a monohydroxy derivative of sulfapyridine and its glucuronide has been definitely established (100 to 103). After feeding *p*-hydroxybenzenesulfonamide to rabbits its glu-

curonide was isolated from the urine (104). Some oxidation to pyrocatechol-4-sulfonamide also occurred. No increase in glucuronic acid excretion occurred after administration of *p*-hydroxybenzenesulfonic acid or of several amino- and hydroxy- substituted derivatives (104). Sulfathiazole also appears to be oxidized to a monohydroxy compound which yields a glucuronide in rabbit urine (105). The glucuronides of *o*-, *m*- and *p*-aminophenol, 3-amino-6-hydroxybenzenesulfonamide and of 3-amino-4-hydroxybenzenesulfonamide were isolated from rabbit urine after feeding the parent compounds (106).

The fate of sulfadiazine is questionable. While acetylation definitely occurs, the acetyl derivative does not give any color with the thiobarbiturate reagent of Kohn (43) so that oxidation to a hydroxy compound and glucuronide formation may also be involved (107). *p*-Chloro-*m*-xylenol administered to human subjects resulted in an increased excretion of glucuronic acid and ethereal sulfate (108). Rat liver slices were observed to conjugate benzoic, *p*-amino- and *m*- and *p*-hydroxybenzoic acids with glucuronic acids. This is in accord with experiments by the same author *in vivo* (109). From analyses of the urine after the administration of a series of morphine derivatives to dogs it was concluded that conjugation occurs on either the phenolic or alcoholic hydroxyl group. Proportional rises in glucuronic acid excretion with morphine dosage suggest that conjugation occurs either with glucuronic acid or its lactone (110). This is in accord with the findings of Gross & Thompson (137). Gross has also observed that dogs poisoned with carbon tetrachloride excreted less conjugated morphine and more in the free state than did normal dogs (138).

*Steroid conjugates.*—The literature concerning steroid conjugates is too extensive to be dealt with comprehensively here. Much of the subject has been reviewed by Venning (111) and elsewhere in these reviews. The structure of the urinary pregnanediol glucuronide has been definitely established as pregnanediol-3, $\beta$ ,*d*-glucuronide (112, 113). A method for the determination of this substance in urine has been described (114) and used widely since. There also appeared a method for the measurement of conjugated estrogens in serum (115). The list of steroid conjugates obtained from urine (estriol and pregnanediol glucuronides, estrone sulfate) has been extended to include stilbestrol glucuronide (116) from rabbit urine after stilbestrol administration; dehydroisoandrosterone sulfate (220) and androsterone sulfate (117) from the urine of a man with an interstitial tumor of the testis. It is noteworthy that the latter compound is 1 to 2 per cent as

active biologically as the parent compound. The inactivation of administered steroid hormones has been shown to occur in the liver and probably involves oxidative processes as well as conjugation (118 to 121).

*Sulfates.*—A micromethod for the estimation of ester sulfate in biological material (blood and pus) was described by Norberg (122). Schmidt offered a method for the determination of free, total, and conjugated phenols and aromatic hydroxy acids (123), while Deichmann (124) described a method for the estimation of free and conjugated phenols in human tissues. The administration of epinephrine was found to augment markedly organic sulfate excretion, but the nature of the conjugated material was not established (125). No increase in glucuronide excretion was noted. Normal blood and tissues of rabbits contained insignificant amounts of phenol in any form. Three minutes after giving the minimum lethal dose it was found in all tissues in both forms. Within twenty-four hours after giving one half of the minimal lethal dose, 75 per cent was found in the urine of which about one half was in the free form and the rest was present as the various conjugates. However, when the minimal lethal dose was given, about 50 per cent appeared to be destroyed within four hours (126).

Arnolt & de Meio studied the production of phenol sulfate by rat tissue slices *in vitro* (127). They found that intestine was more active than liver, while muscle, diaphragm, and kidney were inactive. The respiration of all these tissues was inhibited at phenol concentrations greater than 80 mg. per cent. Later (128) they found that while no increment in oxygen consumption was apparent during this process, it nevertheless required normal tissue oxidation, since no conjugation occurred in an atmosphere of nitrogen or in the presence of cyanide. It did not appear to be necessary to add inorganic sulfate to the medium. Bernheim & Bernheim (129) found that while phenol disappears when incubated with guinea pig liver slices aerobically, only 20 to 30 per cent of that which appears can be accounted for as conjugated. Neither system worked anaerobically. However, they did observe that inorganic sulfate was necessary for ethereal sulfate formation and that under the conditions used methionine and cystine did not produce sufficient sulfate for this purpose. Cocaine was found to inhibit the disappearance of phenol from the medium but did not affect the formation of phenol sulfate. Presumably it blocked the oxidative pathway (129). This is in contrast to the results of Torda (130, 131), who stated that cocaine in extremely high concentration inhibited the

hydrolysis of phenol sulfate by liver slices. She also observed low excretion of phenol sulfate in cocaineized cats, but it is noteworthy that the same animals excreted very little free phenol as well. de Meio & Arnolt repeated their previous observations on the sulfate requirement for phenol conjugation by liver slices and found that while the rat strain they had used in Argentina required no sulfate, livers from Vanderbilt strain rats in the United States only conjugated phenol when inorganic sulfate was added to the medium (132). Pertinent to this discussion is the observation of Borek & Waelsch that only 2 per cent of the sulfur of methionine incubated with liver slices aerobically at a concentration of 290 to 900 mg. per cent was oxidized to sulfate (133), in agreement with early findings by Pirie (134). No extra sulfate was found in rabbit urine after feeding thiourea (135). The origin of the 25 gm. of *p*-cresol isolated by Campbell & Hey from 10 gallons of stallion urine is not evident (136).

A comprehensive study of the fate of salicylic acid was made by Kapp & Coburn (139). Adolescents and adults were found to excrete 80 per cent of a single dose of which 20 per cent appeared unchanged, 55 per cent as salicylurate, 25 per cent as salicylglucuronide, and 4 to 8 per cent as gentisic acid and other oxidised derivatives. Young children and febrile adults appeared to excrete less unchanged salicylate, and fever increased the extent of oxidation and decreased the amount of salicylurate production. Lutwak-Mann (140) isolated gentisic acid from rat urine after the administration of acetylsalicylic acid or of salicylic acid and found that poisoning with carbon tetrachloride or with phosphorus inhibited the formation of the gentisic acid. Hypoprothrombinemia and hemorrhages have been definitely established as resulting from administration of salicylates (141 to 144). Inconclusive evidence was offered that it is the conversion of hydroxycoumarin to salicylic acid which explains the hypoprothrombinemia resulting from the administration of this compound (145). However, after giving sufficient dicumarol to rats to produce clotting times greater than five minutes, no salicylates or their derivatives were found in the urine (146). After feeding vanillin to rabbits, Sammons & Williams recovered 69 per cent as vanillic acid, of which 44 per cent was free and 25 per cent was conjugated. The conjugated material was partly glucuronide and partly ethereal sulfate. The remainder of the dose was recovered as free vanillin, its glucuronide, and ethereal sulfate (147).

*Mercapturic acids.*—Some of the studies on mercapturic acids have already been mentioned above. Stekol isolated *p*-bromobenzylmer-

capturic acid from the urine of rats fed *p*-bromobenzyl bromide, *S-p*-bromobenzyl-*l*-cysteine, and *S-p*-bromobenzylglutathione. He also observed that the inhibition of rat growth produced by *p*-bromobenzyl bromide could be alleviated by the inclusion in the diet of glutathione, cysteine, or methionine (148). This author also found that the rate of growth of rats maintained on diets of varying casein content, with and without bromobenzene, was correlated with the urinary cysteine output as mercapturic acid and the amount of organic sulfur of the diet which still remained for growth purposes (149). The assumption of White & Jackson (150) that bromobenzene inhibits rat growth by inducing cysteine deficiency created by the excretion of mercapturic acid has, therefore, been substantiated by experimental evidence. This technique was also used by West & Jefferson who found that both cysteine and methionine alleviated the growth retardation induced by feeding biphenyl or chrysene (151). Zbarsky & Young (153) described the synthesis of *S*-phenyl-*l*-cysteine and its acetyl derivative and isolated the latter from the urine of rats fed the former (154). These authors also increased the number of known halogenated mercapturic acids by isolating *p*-fluorophenylmercapturic acid after feeding fluorobenzene (156) or synthetic *p*-fluorophenyl-*S-l*-cysteine (156). Blood & Lewis (157) could find no mercapturic acid or extra sulfate in urine after feeding *S*-carboxymethylcysteine. Stekol found no mercapturic acid but 70 per cent of the theoretical amount of hippuric acid after administering dibenzyl thioether to rats (158). He has also observed that  $\alpha$ -hydroxy- $\beta$ -benzylthiopropionic acid and  $\alpha$ -hydroxy- $\beta$ -benzylthiobutyric acid are excreted unchanged by rats and concluded that the  $\alpha$ -hydroxy acids corresponding to amino acids are not automatically oxidized or aminated (159).

*Methylation.*—Much impetus has been given to the study of methylation as a detoxication mechanism by the many studies on the normal metabolic process of transmethylation (160). It has long been known that pyridine and nicotinic acid are excreted in part as methylated products. Knowledge of the physiological significance of nicotinic acid stimulated interest in its metabolic fate. Several methods for the estimation of trigonelline have appeared (79, 161, 162). While quantitative recoveries of nicotinic acid as its methylated product or as nicotinuric acid have only been observed in dogs saturated by long dosing with nicotinic acid or its amide, it was noteworthy that the excretion of what was thought to be trigonelline was greater after a dose of nicotinamide than after nicotinic acid (78, 80, 82). Meanwhile,

Najjar and collaborators (164, 165) observed that the excretion of a substance, designated by them as  $F_2$ , was smaller in the urine of pellagrins and blacktongue dogs than in the urine of normals, and was increased after dosage with nicotinic acid (164). This substance exhibits fluorescence when extracted with butanol from strongly alkaline urine, Huff & Perlzweig isolated a picrate of this compound from human urine collected after ingesting large doses of nicotinamide, and found it to be  $N^1$ -methylnicotinamide, a quaternary base (166). Several modifications of the original Najjar method (164) of determining this derivative have appeared (167, 168, 169). It now seems that the greater part of what was originally estimated as "trigonelline" in urine was actually  $N^1$ -methylnicotinamide. The synthesis of this compound by rat liver slices from nicotinamide and methionine has been demonstrated (170).

In a preliminary publication, Stekol described an inhibition of the growth of the male rats on low protein diets to which 1 per cent nicotinic acid was added and found that methionine, choline plus homocystine, and choline plus cystine, but not choline, homocystine or cystine alone were capable of alleviating the inhibition (171). No complete report has been published, so the author's interpretation of these data are lacking, nor can one explain the failure of nicotinic acid to affect the growth of female rats. Independently, Handler & Dann studied the same problem (3). They observed no effect with nicotinic acid but found that nicotinamide significantly depressed the growth of rats of both sexes. This difference between nicotinic acid and its amide was correlated with the greater excretion of methylated products when the amide was fed. Again, the inhibition was found to be prevented by methionine, choline plus cystine, or choline plus homocystine, but not by cystine, choline, or homocystine alone. At lower levels of nicotinamide feeding they observed marked fatty infiltration of the liver which could be prevented by methionine, betaine, or choline. The ability of choline to prevent fatty livers, while ineffective in promoting growth under these conditions, was taken as evidence that the "fatty livers induced in rats by methionine or choline deficiencies should be ascribed to a deficiency of choline or its precursors rather than to a deficiency in 'labile methyl groups' unless the latter be understood in a very strict sense, *viz.*, choline precursors." The failure of choline to affect the growth rate under these conditions suggested that "either homocystine does not arise in methionine demethylation or, that if it does occur, the mechanism for resynthesis to available methi-



onine is not efficient. The presence of large amounts of dietary cystine may effect the existing equilibria in favor of the existence and remethylation of homocysteine." The same authors also found that while glycocyamine feeding produces fatty livers with low choline content (4), nicotinamide feeding had no effect on the liver choline concentration (3).

Thus by feeding substances which are "detoxified," deficiencies of glycine (and its precursors), cystine (and its precursors), and of methionine and choline have now been produced. It is usually difficult to determine whether growth inhibitions produced in this manner are alleviated, as they are, because a deficiency has been corrected. Stekol (149) has clarified this problem with respect to mercapturic acid synthesis (see above). Moreover, nicotinamide has been found to be non-toxic (i.e., has no effect on growth or liver fat) for rabbits or guinea pigs, both species which do not methylate nicotinamide (172). This may be taken as evidence that the growth failure in nicotinamide feeding is due entirely to the synthesis of N<sup>1</sup>-methylnicotinamide, and that this procedure is tantamount to feeding a choline- and methionine-poor diet, adequate in the other dietary protein components. It affords an example of a situation in which an apparently innocuous compound undergoes a classical "detoxication" process with the production of a dietary deficiency.

Selenite has been found to be less toxic to rats on a high protein than on a low protein diet (173). Cystine possessed but slight growth accelerating action and methionine much more so. No data were given concerning the excretion of the proposed dimethyl selenide under these conditions. Most of the urinary selenium excreted by rabbits fed selenized wheat appeared in the ethereal and neutral sulfur fractions and 15 per cent as inorganic selenium; none was found in the bromophenylmercapturic acid isolated after feeding bromobenzene (174). The excretion of selenium as volatile respiratory products by rats after the subcutaneous injection of sodium selenate containing radioactive selenium was demonstrated by McConnell (175), without chemical identification of the product.

The possibility of physiological demethylation (or dealkylation) of abnormal metabolites has received but scant attention. While the demethylation of sarcosine (68, 70) by rat liver is accomplished by oxidation of the methyl group (69), the N-methyl derivatives of methionine, alanine, leucine, and histidine were demethylaminated to the  $\alpha$ -keto acids by rat kidney and liver preparations (176). While

N-ethylglycine is slowly oxidized by rat liver (69), it does not appear to yield extra glycine for hippuric acid synthesis (177) in the rabbit. N-methyl- and N-ethylbarbital gave rise to barbital in the urine of dogs after administration, while longer alkyl substituents were removed to a slight extent only (178). After feeding *p*-dimethylaminoazobenzene to rats, *p*-aminophenol and *p*-phenylenediamine and their acetyl derivatives were found in the urine (179), while liver brei converted the dye to *p*-phenylenediamine (180). The possibility of physiological availability of the methyl groups of this compound has been suggested by the finding that its inclusion in the diet prevented the hemorrhagic kidneys expected of choline deficient young rats (181).

*Oxidations and reductions.*—Considerable interest has been evinced in the fate of hydrocarbons. Stetten (182) has shown that the paraffin, n-hexadecane is well absorbed by the rat intestine in moderate amounts and that it is in part deposited in the fat depots as such and in part oxidized to fatty acids. Bernhard & Gressly (183) definitely established that benzene can be oxidized by way of muconic acid. They injected 1.0 to 1.5 cc. of  $C_6D_6$  into rabbits and isolated from the urine of one animal about 24 mg. of muconic acid containing 7.2 atom per cent deuterium while the benzene originally given contained 10.7 atom per cent deuterium. They also found that muconic acid cannot be recovered in any appreciable quantity when given directly to rabbits, thus accounting for the poor yield after benzene administration. Using sheep as subjects, Harvey *et al.* (184), on examining urine found cumic acid after giving *p*-cymene; phellandric acid, cymene and carvotanacetone after giving  $\alpha$ -phellandrene; and thymol and carvotanacetone after giving piperitone. After administration of 3,4-benzopyrene the stools of mice and rats appear to contain both 8-hydroxy-3,4-benzopyrene and 3,4-benzopyrene-5,8-quinone (185, 186, 187). The administration of 1,2,5,6-dibenzanthracene results in the appearance of the corresponding 4',8'-dihydroxy compound in the urine and stools of rats and mice and perhaps a monophenolic compound in rabbit urine (188, 189). It is noteworthy that while oxidation occurs with both of these carcinogenic hydrocarbons, the resulting phenols do not appear to be conjugated with glucuronic acid or sulfate.

Wick (190) investigated the fate of a series of branched chain aliphatic acids while Carter and his collaborators (75) have studied the fate of a series of phenylated branched chain fatty acids. The results of these studies, while mentioned here, are best considered in the chapters on lipid metabolism found elsewhere in these volumes. Bern-

hard (191) has fed a series of dicarboxylic acids whose aliphatic chains were labeled with deuterium. While succinic acid appeared to be completely oxidized, or otherwise destroyed, adipic, suberic, and sebacic acids were excreted in large measure, unchanged. This is in agreement with the studies of Emmrich *et al.*, on alkyl substituted dicarboxylic acids (192). Methyl formate has been found to increase the rate of respiration of liver, kidney, spleen, muscle, and brain slices as well as that of several types of sarcoma tissue while this does not occur with either methyl alcohol, formic acid, or a mixture of these (193). Hoff-Jorgensen has compared the oxidation by pig heart preparations of the various 1,2-dihydroxybutyric acids (194). In increasing order of ease of oxidation they were: *l*-erythro, *d*-erythro, *d*-threo, and *l*-threo dihydroxybutyric acids. *d*-Amino acids appear to give rise to more urinary ammonia after administration than do *l*-amino acids (195). Rabbits appear to be unable to metabolize  $\beta$ -amino acids since  $\beta$ -amino-valeric, -caproic, -octanoic, and -decanoic acids, were all excreted unchanged by these animals (196). After feeding *d*-mandelic acid, Montenbruck (197) found that about 65 per cent was excreted unchanged, 14 per cent appeared as *l*-phenylaminoacetic and 14 per cent as benzoylformic acid, and the remainder as hippuric acid. On the other hand after a dose of *l*-mandelic acid 73 per cent appeared in the original form, 7 per cent as benzoylformic acid and the rest as hippuric acid. The large excretion of the unchanged material is compatible with the therapeutic use of mandelic acid as a urinary antiseptic.

$\beta$ -Naphthylamine administered to rats, rabbits, and monkeys is in part excreted in the acetylated form and in part as the N-acetyl-6-hydroxy derivative. Since the amount of phenol increased after acid hydrolysis of the urine, apparently there occurred some conjugation with glucuronate or sulfate in contrast to the hydrocarbons discussed above (198). Gad has found that  $\beta$ -phenylisopropylamine (benzedrine) is readily destroyed by liver tissue (199). Beyer studied the fate of a large series of sympathomimetic and related amines (200 to 204), when given to men and dogs and when incubated with amine oxidases and polyphenol oxidases and ascorbic acid. A detailed account of this author's conclusions concerning the relationship between molecular configuration and rate of oxidation cannot be presented here for lack of space.

*Alcohol.*—Westerfeld *et al.* found that the disappearance of orally administered ethanol from the blood serum of dogs was accelerated by feeding pyruvate (205) but no effect was noted by Gregory *et al.* when

the alcohol was given intravenously and pyruvate was introduced by vein or stomach tube (206). This suggested the possibility that, after oxidation to acetaldehyde, condensation with pyruvate might be effected with a simultaneous production of acetoin and carbon dioxide. However, since acetaldehyde disappears from the circulation quite rapidly, and since the simultaneous administration of alcohol with pyruvate or lactate results in an increase in blood acetaldehyde, it was concluded that there must exist a system in which oxidation of alcohol is coupled with reduction of pyruvate (207). When acetoin was given to dogs 5 to 25 per cent of the dose appeared in the urine as 2,3-butylene glycol; the remainder was apparently oxidized (208). These studies were repeated by Greenberg, who described a new method for the determination of acetoin (209), but who could find no acetoin in serum after administration of alcohol with pyruvate (210). He also demonstrated that acetoin is about 25 per cent more toxic than is alcohol for rats and produces the same symptoms (5). In contrast to these studies, Bernhard (11) found that when deuterium labeled alcohol was administered with sulfanilamide to rats, 24 per cent of the acetyl groups in the urinary acetylsulfanilamide appeared to originate in the dietary alcohol. Since he had also shown that this acetylation directly employs acetic acid (see above), he believes that alcohol is oxidized stepwise to acetaldehyde and acetic acid. The specificity of the alcohol oxidase found in liver brei was studied by Bernheim & Handler (211). While these preparations oxidized ethylene chlorohydrin, ethylene glycol and  $\beta$ -hydroxypropionitrile somewhat more slowly than ethyl alcohol, no oxygen uptake was observed with glycerol, ethanolamine, or ethylene-bromohydrin. Propylene glycol was observed to disappear from the medium when perfused through cat liver although the actual oxygen uptake of the liver is decreased; there appeared a simultaneous increase in the glycogen and lactic acid content of the medium; addition of insulin accelerated all phenomena involved except the appearance of lactic acid (212). Roe (213) claims that the symptoms of methyl alcohol poisoning are due to the production of formic acid which in turn inhibits iron-containing respiratory systems, resulting in the accumulation of lactic acid. The amblyopia of this state was thought to be referable to the interference by formic acid with the oxidative processes of the retinal cells. Scott (214) observed an increase in the acetaldehyde and nitrite content of the blood of rabbits after administration of nitroethane.

After feeding 85 gm. of  $\beta$ -ionone to rabbits, their pooled urines

were found by Bielig & Hayasida to contain  $\beta$ -ionol and dihydro- $\beta$ -ionol equivalent to 42 per cent of the original doses although no tetrahydro derivatives were found (215). The remainder of the material was accounted for as benzoic and *m*-hydroxybenzoic acids. After administering 18 gm. of benzalacetone,  $C_6H_5CH:CHCOCH_3$  to rabbits, Fischer & Bielig (216) found in their urine 5 gm. of  $\beta$ -phenethyl methyl-carbinol,  $C_6H_5(CH_2)_2CHOHCH_3$ , 1 gm. of benzal dimethyl-carbinol,  $C_6H_5CH:CHCHOHCH_3$ , and 1 gm. of 2,4-dihydroxy-4-phenylbutane,  $C_6H_5CHOHCH_2CHOHCH_3$ . Cinnamic alcohol,  $C_6H_5CH:CHCH_2OH$ , gave rise to benzoic acid,  $C_6H_5COOH$ , and cinnamic acid,  $C_6H_5CH:CHCOOH$ , while  $\alpha$ -ethylcinnamic aldehyde,  $C_6H_5CH:C(C_2H_5)CHO$ , yielded  $\alpha$ -ethylcinnamic acid,  $C_6H_5CH:C(C_2H_5)COOH$ , in the urine. The urinary products after giving geraniol,  $(CH_3)_2C:CH(CH_2)_2C(CH_3):CHCH_2OH$ , were found to be two dibasic acids,  $HOCC(CH_3):CH(CH_2)_2C(CH_3):CHCOOH$  and  $HOCC(CH_3):CH(CH_2)_2CH(CH_3)CH_2COOH$ . These should be considered in the light of the extensive studies of Carter mentioned above (75).

Bernheim (217) observed what may be the first step in the conversion of neoprontosil to sulfanilamide, the reduction of the azo linkage by liver brei *in vitro*. Kohl & Flynn (218), studied the metabolism of the various nitrobenzoic acids. Liver and kidney brei were found to reduce the *m*- and *p*- compounds to amino groups while the *o*- compound was relatively resistant. When the *m*- and *p*- compounds were given orally to rats, their urine appeared to contain almost all the possible intermediates, including nitroso and hydroxylamino compounds. All of the amino compounds appeared in the urine as their acetyl derivatives. This reduction had also been observed by Miller (220). In like fashion, *p*-nitrobenzenesulfonamide was found to be reduced to the corresponding amino compound by rat liver brei and to yield the amino and acetylamino compounds in rat urine after oral administration (219) while 2-(*p*-nitrobenzenesulfonamido)-pyridine has been shown to be reduced to sulfapyridine in the rat intestine, presumably by intestinal bacteria (221, 222). Azobenzene fed to rats yielded aniline in the urine, as well as a substance which could be converted to benzidine on treatment with acid followed by alkali (223). Channon *et al.* (224) administered to rabbits 2,4,6-trinitrotoluene and isolated from the urine the 2,5-dinitro-4-hydroxylamino-, 2,6-dinitro-4-amino-, and 2,4-dinitro-6-amino derivatives of toluene. The 2,2',6,6'-tetraamino-4,4'-azoxytoluene previously reported by others as present in the urine

of patients with trinitrotoluene poisoning, was shown to be absent in fresh urine, and was claimed to be formed by oxidation in air during the extraction procedure. The above reductions of trinitrotoluene are apparently mediated by the succinoxidase system (224). Phenothiazine administered to sheep gave rise to thionol and phenothiazone in the urine (225).

*Indoxyl formation.*—A method for the determination of indoxyl compounds in urine depending on the color produced with thymol was described by Meiklejohn & Cohen (227). Stoppani investigated the formation of indoxyl (228) and of indoleacetic acid (229) from numerous nitrated aromatic precursors. The same author reported that the conversion of indole to indoxyl in frogs and toads was impaired by hepatectomy (230). Nicolai (231) stated that during the process of indole formation only small amounts of indican were made in the intestinal wall, with the bulk of this synthesis occurring in the liver. This is in agreement with the observations of Baroc that hepatectomized dogs excrete less indoxyl derivatives than do the same animals before hepatectomy (232). In quantitative studies Böhm measured glucuronide and sulfate excretion after administering a number of compounds. He found that indole, indene, hydrindene, and triketohydrindene all caused increased excretion of sulfate and glucuronide. Cumarone,  $\alpha$ -,  $\beta$ - or diketindole, all increased sulfate excretion without affecting glucuronide excretion, while thionaphthene increased only the glucuronide fraction (233, 234, 235).

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# THE BIOCHEMISTRY OF MALIGNANT TISSUES

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## INTRODUCTION

Over seventy-five years ago, chemical physiologists, led by Hoppe-Seyler, Kossel, and others, had hoped that problems in tissue function might be partially explained in terms of the properties of isolated chemical components. From the chemical characterization of normal function to the development of a chemical pathology seemed the next logical step. In a letter to His, dated 1890, the great Swiss physiologist, Friedrich Miescher, summed up this feeling and expressed the belief that the study of the chemical phenomena of tissues would clarify many pathological manifestations, such as inflammation and degeneration (cancer) which were obscure or incapable of explanation by microscopic examination alone, and he concluded the letter with the rueful comment, "Das Mikroskop lässt einen . . . gewiss oft im Stich" (1). There is no doubt that well over fifty years ago progressive (if cloistered) medical opinion was seeking a more dynamic approach to problems in pathology than those afforded by the classical techniques.

It is interesting to note, however, that in the field of experimental cancer research the services of biochemistry were enlisted only about two decades ago, and this introduction was accelerated by the discovery that chemical substances could cause cancer. The resources of biochemistry have thus only recently emerged as a mode of attack on one of the most baffling of medical and biological problems. Too often, however, the employment of these resources has been incidental to the spare time of the busy clinician or of the occasionally curious and isolated biochemist. The systematic application of biochemical techniques to the cancer problem over a long term period by professional investigators experienced in both fields is a still more recent development. This development, encouraged by enlightened opinion largely in the United States and stimulated to action by the recognized limitations of surgery and radiation, offers the possibility of initiating powerful, diverse, and sustained lines of approach toward the elucidation and ultimate control of this mysterious cellular derangement.

In their comprehensive review in last year's *Annual Review of*

*Biochemistry*, Burk & Winzler covered much of the biochemical literature of cancer for the past few years and considered several of the controversial matters in detail (2). Furth has recently reviewed the biological factors concerned in neoplasia (3), Rusch has reviewed certain aspects of carcinogenesis (4), and Burk & Winzler (5), and Dodds (6) have considered the influence, respectively, of nutrition and of hormones. In the symposium monograph *Cancer* (based on the conference held at Gibson Island in 1944) and published by the American Association for the Advancement of Science, Washington, D.C. (1945), are to be found fairly full accounts of many of the frontier lines of investigation in cancer research now going on in the United States.

This reviewer has undertaken the task of extracting from the literature those data which appear to be significant and eminently reasonable, and of writing, primarily for the information of biochemists not in this field, a brief outline of the principal contemporary developments. This outline is designed merely as a brief assessment of what the biochemical approach to the cancer problem has so far achieved and as an illustration of the possibilities of the reciprocal enrichment of the fields of normal and of abnormal physiology. A good deal of data has been necessarily omitted, some with deliberation and some with regret (and some no doubt by inadvertence and faulty judgment); and if that which is spectacular also shares this fate, the reviewer can only plead that he has heard the cry of wolf too often.

The topics are considered in the following order: (a) the induction of tumors, or carcinogenesis, (b) the attempts to influence the induction of tumors, or the growth rate of established tumors, by means of various dietary regimes, alterations in endocrine status, and chemotherapy, and (c) the chemical characterization of tumors. These topics are by no means sharply separable.

#### CARCINOGENESIS

*Extrinsic agents.*—With the publication by Cook, Kennaway, and their associates, of the sixth and apparently concluding paper in their series of studies on the production of cancer by pure hydrocarbons (7), a brilliant chapter in the history of experimental cancer research drew to a close. Begun two decades ago with the arduous task of isolating the active carcinogenic fraction from coal tar, discovering in the interim the first pure chemical (1,2,5,6-dibenzanthracene) to produce cancer in an animal, and ultimately isolating from tar the hitherto

unknown and powerful carcinogenic agent, 3,4-benzpyrene, the program of the London group eventually embraced the study of the carcinogenicity of literally hundreds of synthetic polycyclic hydrocarbons and their derivatives and furnished one of the most striking examples of co-operation among scientists of different disciplines. In 1932 a group of investigators in Boston, led by Shear and Fieser, began a series of similar studies on the carcinogenicity of the polycyclic hydrocarbons. For a decade on both sides of the Atlantic, this program was actively pursued and, except for minor points of difference, with complete accord. A general assay of the approach and its results has been written by Shear (8). It is only fitting at this time to recall with appreciation the accomplishments of these two groups of investigators.

The positive general findings included the discovery of pure carcinogenic agents which under suitable modes of administration could produce certain tumors at will in mice. The London workers used heterozygous mice with maximal doses of carcinogens applied chiefly to the external skin. This procedure was satisfactory for their purpose which was primarily a study of the relation between chemical structure and cancer reaction. Shear adopted a more biological approach and employed mice of pure strains, a practice which introduced a necessary genetic control. The active hydrocarbons administered under the skin or inserted as cholesterol pellets within certain tissues (9) revealed the high degree of susceptibility of connective tissue toward these agents. Large proportions of the tumors so elicited in the earlier experiments were therefore sarcomas. When the techniques of administration of the hydrocarbons were still further widened by others and applied to a greater number of pure strains of mice, it was found that the intravenous administration of fine emulsions of active hydrocarbons gave rise to pulmonary tumors (10, cf. 11), the inclusion of such emulsions in the drinking water gave rise to adenocarcinomas of the small intestine (12), the implantation of such emulsions into the wall of the pyloric stomach induced the formation of gastric adenocarcinomas (13), and the painting of skin gave rise to leukoses (14).

A tissue largely resistant to the effect of the active hydrocarbons has been the liver (9). The induction of liver cell tumors has been effected by the use of another type of carcinogen, the azo dyes in specific configuration. This development is due to the observations of the Japanese workers, Yoshida (15) and Kinoshita (16), who found respectively that *o*-aminoazotoluene and *p*-dimethylaminoazobenzene



when included in the diet could induce the formation of hepatomas in rats. These findings were confirmed by others, and it was noted that although the feeding (17) and subcutaneous injection (18, 19, 20) of the former substance could induce hepatomas (as well as pulmonary tumors and hemangioendotheliomas) in certain strains of mice, similar administration of the latter substance failed to affect the livers of such mice more than slightly (17, 20). With the finding by Edwards *et al.* (21, 22) that the feeding of carbon tetrachloride could produce hepatomas in susceptible strains of mice, yet another carcinogenic agent for the hepatic cell was discovered. Histologically, as well as chemically (*vide ultra*), the hepatomas arising in any one strain spontaneously or through induction by *o*-aminoazotoluene or carbon tetrachloride are frequently indistinguishable.

The observation that the same kind of tumor can arise through any one of a number of entirely unrelated inciting agents is by now an accepted fact and could hardly have been foreseen. Sarcomas may arise by such varied means as ultraviolet irradiation (4, 23), the implantation of bakelite disks (24), and the subcutaneous injection of thorium oxide (25), of methylcholanthrene (7) or of *p*-dimethylaminoazobenzene (20). Pulmonary tumors can arise by injection of urethane (26), methylcholanthrene (10), or aminoazotoluene (20). Chemically dissimilar induction agents for the hepatomas are referred to above. When to these agents such endogenous factors as viruses and hormones are added, it is readily appreciated that an embarrassing richness of carcinogenic agents apparently exists. The explanation of how such diverse factors can effect the production of the same kind of tumor is hardly available at the present time. The mechanism of carcinogenesis is yet obscure; and although it had been hoped that the determination of the specificity of carcinogenic agents of closely related chemical structure would prove enlightening, it must be admitted that since bakelite disks and ultraviolet radiation both induce sarcomas, the sharp difference between the potency of isomers of methylcholanthrene no longer seems crucial.

It is interesting to note, however, that attempts have been made to elucidate the mechanism of the hepatoma genesis of *p*-dimethylaminoazobenzene by a study of the effect of certain of its possible metabolic products on various hepatic enzyme systems (27, 28). Interesting results on the metabolism of the dye have emerged, of which those by Miller & Baumann (29) have been indeed noteworthy. It may be wondered whether the very metabolic reactivity of the dye

is not a false clue to its potency. It is conceivable that noncarcinogenic isomers of the dye could yield the same products, especially if demethylation is the primary metabolic step. A better approach might be to eschew the tempting and possibly deceptive reactive carcinogens and to turn instead to a consideration of the behavior of the apparently chemically inert agents such as carbon tetrachloride (30).

The sum contribution of the workers in this field has been far more than the mere supply of chemical agents which will produce tumors at will, or the possible clarification of several practical problems involved in the genesis of human occupational cancers. If the wide array of carcinogenic agents appears to make the early interpretation of carcinogenic mechanism an apparently discouraging task, nevertheless there have emerged from the work in this field profoundly important implications not only for measures of cancer control but also for the integration of normal tissue mechanisms. The use of inbred strains of mice has revealed a connection between genetics and susceptibility to cancer (31, 32, 33). The use of the azo dyes (19, 20) and the use of benzpyrene in marginal doses (34), applied to inbred strains of mice, have revealed the marked influence of sex on cancer susceptibility in nonsexual tissues, as liver, lung, and subcutaneous fibroblasts; such findings suggest the influence of hormones on the integration of normal tissue function and antedate the recent striking observations of the relation between hypophyseal secretions and lymphoid tissue (35). The variation in the nature of the menstruum by which the carcinogen is applied, i.e., by altering the fatty vehicle of the hydrocarbons (4, 34, 36, 37, 38) or the alteration of the components of the diet with which the azo dyes are mixed (5) (*vide ultra*), showed even in the earlier experiments the possibility of markedly influencing the degree and rate of response of the same tissue in the same strain of animal to the same agent. These and later findings have suggested methods of control through techniques of genetics, endocrinology, nutrition, and chemotherapy. Thus the original intent of this field, which was to illuminate the mechanism of carcinogenesis by a study of chemical structure, and which had only limited success, has evolved instead into the pursuit of ever-widening and productive areas of investigation, areas which could hardly have been earlier envisioned.

Emphasis has been placed on the problems of the genesis of induced tumors, but it is obvious that the areas of investigation also embrace similar problems of spontaneous tumors, which for all purposes may

be considered to be tumors induced by intrinsic factors known or unknown. Frequently the administration of carcinogens merely hastens the appearance and increases the incidence of tumors in certain strains at certain sites which would naturally appear later in the life of the animals (10, 39, 40, 41). This raises the question whether all tissues are not potentially foci of spontaneous malignancy of varying threshold which is lowered by the effects of the carcinogen. No distinction will therefore be drawn between induced and so-called spontaneous tumors. The techniques of genetics are outside the scope of this review. Before considering the other techniques, a brief survey of the carcinogenic factors of intrinsic origin follows.

*Intrinsic agents.*—A distinction in certain cases between extrinsic and intrinsic agents may be somewhat arbitrary. Although a polycyclic hydrocarbon such as methylcholanthrene has been considered as an extrinsic agent, it is possible to conceive of this substance as arising by a process of abnormal metabolism from the bile acids (42), and hence under such conditions to be classed as an endogenous agent. In this way, a possible explanation for the origin of certain spontaneous tumors may be advanced. On the other hand, there are agents which are known without doubt to be elaborated by the organism, and these are the sex hormones and the viruses.

From the time of the classical experiments of Leo Loeb and of Lacassagne (cf. 43), it has been recognized that the sex hormones are involved in the genesis of tumors of sex tissue. The fact that such tumors may be induced by administration of pure hormones (39, 44, 45, 46) has frequently been demonstrated. For a masterly summary of this field, the reviews by Loeb are suggested (43, 47).

The inbreeding of many generations of mice resulted in the development of genetically pure lines, some of which, like the C<sub>3</sub>H strain, show a high incidence of spontaneous mammary carcinoma in both virgin and bred mice, some like the C57 black strain show very little incidence whether virgin or bred, and some like the A strain show a much higher incidence in bred than in virgin mice. The mice in each strain, kept under identical conditions, receive the same food, and possess apparently the same hormones. The explanation for the observed differences, once thought to be primarily genetic, was elucidated by the staff of the Jackson Memorial Laboratory (48), by Bittner (49, 50), and by Andervont (51). When the newborn of C<sub>3</sub>H mice were foster-nursed by C57 black mothers, or vice versa, the mammary tumor incidence of the fostered mice approached that of the

strain of the foster mother. Genetic and hormonal factors alone were obviously unable to account for this phenomenon. It became apparent that there was present in the milk of the  $C_3H$  strain an inciting agent for mammary tumor formation which was absent from the milk of the C57 black strain. In the absence of this factor, few tumors would arise in a strain ( $C_3H$ ) otherwise susceptible. However, in the A strain, which possesses this inciting factor in the milk, mammary tumors arise chiefly in bred females; there is demonstrated in this case the additional requirement of hormonal stimulation. On the basis of these findings, it has been concluded that at least three factors are involved in the occurrence of mammary tumors in mice, (a) genetic susceptibility, (b) the inciting agent in the milk, and (c) hormonal stimulation (50). All three factors are inherent in the animal. The milk factor from  $C_3H$  mice introduced into otherwise unsusceptible C mice persists for succeeding generations (52). Such foster-nursed mice treated with synthetic estrogens readily produced mammary tumors, whereas  $C_3H$  mice originally foster-nursed by a low tumor line gave rise to few tumors; there is apparently a need for the interrelated action of hormone and milk factor for the production of mammary tumors (50, 53).

The inciting agent for mammary tumors present in the milk of certain strains of mice is found as well in other tissues of these strains, notably the spleen (54), thymus (54), whole blood (55), and the mammary gland tumor itself (56, 57). Material of high activity could be sedimented from saline extracts of the tumor (58), and it seemed likely that the tumor-inciting activity was associated with particles having sedimentation constants between 25 and 200 S (59). It is of considerable interest that the inciting agent, from whatever source, could produce tumors when fed to mice sufficiently young, and this fact suggests that the material may be either resistant to the action of the digestive enzymes or else dependent upon them for degradation into active fractions. However, the inciting agent is also transmissible by injection of whole blood, or by implantation or injection of extracts of spleen or thymus. See review by Andervont (60). The fact that relatively little work has been as yet performed on the chemical characterization of the inciting agent is probably due to the lack of a rapid method of bioassay. This transmissible agent in most respects possesses properties generally applied to viruses, and the tentative classification of the milk factor in this category does not seem unreasonable in view of the fact that similar transmissible chemical agents have been

known for some time to produce cancer in chickens and related fowl (61) and in rabbits (62, 63, 64).

The Rous agent which transmits chicken sarcoma and the Shope agent which transmits papillomas in cottontail rabbits are apparently complexes of lipid, nucleic acid, and protein, with sedimentation constants respectively of 550 S (65) and 275 S (66). The chemical characterizations of these agents have recently been reviewed (67) and need not be further discussed here. The Shope agent produces benign papillomas in the wild rabbit; introduced into domestic rabbits, the induced papillomas ultimately become highly malignant and the transmissibility of the agent coincidentally disappears. The alteration of the benign into the malignant tumor is greatly hastened by the simultaneous administration of such hydrocarbons as methylcholanthrene (62, 63, 64). By themselves, the hydrocarbons applied to the skin of the domestic rabbit induce only scanty, benign lesions within this experimental period.

These findings illustrate again that the carcinogenic potency of a given substance may be markedly affected by the administration of substances which are themselves either very weakly carcinogenic or noncarcinogenic, which need bear little or no chemical relation to the specific carcinogenic agent, and which may be applied subsequent to that of the agent and at a site far removed from it (4, 68, 69, 70). Certain of the supplemental materials promote, still others retard, carcinogenesis. In few cases is the mechanism even partially apprehended, and of these, perhaps two examples are (a) the supplementary (or complementary) action of estrogen and the milk factor in the genesis of mammary tumors in certain strains of mice (53), and (b) the inhibitory effect of brombenzene on epidermal carcinogenesis which appears to have its origin in a disturbance of the sulfur metabolism of the skin prior to what would have been the carcinogenic process (71). The net impression obtained from all these studies is that carcinogenesis at any one site may be affected by a number of general systemic factors. These factors may include various dietary essentials, the effect or effects of which may be tested by either withdrawal or excessive application.

#### ATTEMPTS AT CONTROL THROUGH NUTRITION

This subject may be divided into two categories: the effect of diet on tumor induction, whether "spontaneously" or by known carcinogens, and the effect of diet on the growth of tumors already established.

*Induction.*—Under carefully controlled conditions, there is no doubt that the latent period of induction of a wide variety of tumors in both rats and mice can be prolonged by various forms of dietary restriction. These restrictions, in an otherwise adequate diet, may involve caloric intake (72, 73 to 76), certain vitamins of the B group (77, 78), and certain essential amino acids (79, 80, 81). The effect of the dietary deficiencies is apparently to delay and reduce the numbers of tumors, rather than to completely abolish their appearance. Tannenbaum noted that a decrease in tumor incidence occurs when the caloric-restricted diet is instituted at any time in the life-span of the animals prior to the appearance of the tumors; it seems to be true, however, that the earlier such a deficient diet is instituted, the lower is the subsequent incidence of tumors (73). With the introduction of the deficient diet, whether deficient in calories or in an essential amino acid, the further growth of young animals slows down or ceases, and care must be exercised in devising such a diet that the animals at least maintain their weight during the experimental period.

The arrest in body growth of young animals fed the inadequate diet is not *per se* the explanation of why such animals develop fewer tumors and at a later time than do their full-fed, full-grown controls. Weanling mice placed either on a cystine-deficient or on a lysine-deficient diet are equally stunted in growth, but while subsequent painting with methylcholanthrene resulted in a low incidence of leukemia in the mice on the former diet, the incidence of this induced disease in the mice on the latter diet was nearly as high as in the controls (80). Weanling mice maintained at a level of stunted growth by a diet deficient in cystine showed no mammary tumors within a period of twenty-two months, as contrasted with an incidence of 100 per cent in the controls; when, however, the mice on the restricted diet were treated with stilbestrol, the tumor incidence rose to 44 per cent (82). These striking findings raise the issue of whether the dietary deficiency does not reduce tumor incidence by affecting the glands of internal secretion directly, and the tumor genesis thereby only indirectly. Further studies revealed that the mice on the various deficient diets were anestrus, and their mammary tissue failed to grow in the virgin and atrophied in the breeding female; implantation of stilbestrol pellets in such mice resulted in growth of the mammary tissue and initiation of continuous estrus, and, as noted, a rise in tumor incidence (83).

Several investigators have demonstrated that caloric restriction

leads to atrophic changes in glands of internal secretion (84 to 88) and that diets deficient in certain essential amino acids cause failures of pregnancy (89). The lowering of the basal metabolic rate is a familiar sequel to inanition. It would appear that the hormone-producing centers are early casualties of dietary deficiencies. The connection of hormones with tumor genesis has been alluded to above and will be discussed in the following section. The work of the Whites, described in the previous paragraph, has, through nutritional techniques, forged another link in the chain between endocrine status and tumor genesis. A new approach is thereby suggested.

In contrast with various forms of dietary deficiencies which produce a decrease in tumor incidence, the inclusion of fats in high degree in diets apparently causes an increase in such incidence (90, 91, 92). The fatty acids, rather than the glycerol component of the fats, seem to be responsible for this effect (4).

*Growth rate of established tumors.*—From present indications, the effect of dietary alterations on a tumor already established is relatively small. In mice kept for some time on a deficient diet, the tumors which ultimately appear grow at the same rate as tumors in the control group (72, 73, 93). Tumors which are transplanted into mice adjusted to a restricted diet frequently grow as rapidly as in control mice on an adequate diet (94). Tumors may be established and will grow in mice fed a diet negligible in protein, the animals remaining in a state of continuous negative nitrogen balance (95). Under these conditions, the tissues of the host break down to supply the proteins for the needs of the growing, hyperparasitic tumor.

There appears to be a decrease in the growth rate of tumors established in well-fed animals when severe restriction of calories (96) and of riboflavin (97) is imposed, but with the initiation of this restriction the body weight of the animals also falls off, and pathological changes intervene. Little differential effect of tumor weight and body weight is attained. The riboflavin content of the tumor under these conditions is not so rapidly depleted as that of liver and muscle (97). Deprivation of thiamine in originally well-fed tumor-bearing animals produces no decrease in size of the tumors (98). The major effect of dietary deficiencies is on the induction rather than on the growth of tumors. As long as the host is alive its body tissues are a potential source of nourishment for the tumor (*vide infra*), and thus paradoxically the host strips itself to feed that which will eventually destroy it.

## ATTEMPTS AT CONTROL THROUGH ENDOCRINOLOGY

The connection of the sex hormones with cancer of sex tissues is by now an old story (43). The most striking recent work on this aspect has been the clinical observations of the Gutmans (99) and of Huggins and his co-workers on the effects of castration and of stilbestrol administration on the progress of disseminated carcinoma of the prostate (100, 101, 102). This work is a unique merging of the techniques of surgery and of biochemistry. Both normal and malignant prostatic epithelia are very high in acid phosphatase activity (99, 103). In cases of metastatic carcinoma, enough of the enzyme is given off to the blood stream to raise significantly the level of acid phosphatase activity of the serum, and the degree of this increase furnishes an approximate index of the extent and severity of the disease (104). Surgical castration of the patient or chemical castration through administration of estrogen results in a striking remission of the malignant symptoms accompanied by a regression in size of the primary tumor, together with a concomitant lowering of the serum acid phosphatase level toward the normal. The serum acid phosphatase level serves, therefore, as an aid in prognosis. There is little doubt that carcinoma of the prostate is stimulated by androgens and repressed by estrogens. The work has been too recent to gauge its long-term effects, for it is not the fashion in oncology to describe results in terms of cures but only in survival periods.

The connection of the hormones of sex and of ostensibly non-sex glands with the genesis of tumors of non-sex tissue has been demonstrated by interesting newer developments (*vide infra*). Fibroids may be induced in the guinea pig by administration of estrogen and may be caused to regress by subsequent treatment with progesterone (105). Administration of estrogen in certain strains of mice produces a high incidence of tumors of lymphoid (106) and of hepatic tissues (107). Estrogen administration in certain strains of mice produces a marked decrease in the induction period of epidermal carcinoma (108). The high incidence of leukemia in female mice over that of males of the same strain has been related to the inhibitory effect of androgens (109). Thymectomy of mice of a high-leukemic stock resulted in a drastic reduction of the incidence of the disease in both males and females (110). These are only a few of the more striking recent observations. No attempt can be made at present to offer even a partial explanation of such diverse findings. They are mentioned, however,



to illustrate the apparent hormone control of very different tissues, and the obvious fact that sex and other hormones may be involved in the maintenance of non-sex tissues and in their malignant transformations.

#### ATTEMPTS AT CONTROL THROUGH CHEMOTHERAPY

This field may be considered as involving the use of chemicals ordinarily foreign to the animal body, which, when administered to a tumor-bearing animal at a site removed from the tumor, will adversely affect the latter without destroying the former. With few exceptions, the work has been haphazard in the extreme [cf. Dyer (111)] and, with no suitable clues, has been largely a groping in the dark and in the Chemical Formulary.

The most important developments are due to Shear *et al.*, who used purified materials isolated from filtrates of certain bacterial cultures (112), and to Furth and his co-workers, who employed benzene and arsenite (113). The latter group observed that the administration of these chemicals to certain strains of mice inoculated previously with transmissible leukemic cells resulted in a definite prolongation of life of the animals, without, however, effecting a "cure" of the disease. Like the use of benzene and arsenite, that of the bacterial filtrates stems from old clinical observations. In a series of careful, systematic experiments, Shear and his co-workers succeeded in isolating from certain bacterial filtrates a material of high particle weight, heat-labile, chiefly polysaccharide in nature, which, when administered to mice carrying transplanted tumors, effected hemorrhage and necrosis in a large proportion of the tumors without similarly affecting normal tissues. Analogous observations on the hemorrhage-producing capacities on tumors of crude bacterial filtrates have been made by others (114). The systematic study of the effect of such materials on human cancer remains to be performed.

#### CHEMICAL CHARACTERIZATION OF TUMORS

This subject has recently been extensively treated by the reviewer in the symposium monograph of the 1944 conference on cancer held at Gibson Island (115). The salient points of the subject are treated in this section under two headings: (a) the chemical properties of tumors as compared with those of normal tissues, and (b) the chemical properties of the tissues of tumor-bearing animals as compared with those of normal animals.

*Properties of tumors.*—Each normal tissue of an animal, except possibly some rudimentary relics, carries on a specific and unique function. To effect this function, the particular tissue is metabolically (as well as morphologically) differentiated and possesses a chemical structure and a pattern of enzymatic catalysis peculiar to itself. The life of the animal thus represents the harmonious integration of a number of individual metabolic mosaics. Tumors, like the normal tissues from which they arise, possess chemical substances, enzymes, etc., and their existence too must be at least partially explicable in terms of a chemical and metabolic pattern.

The classic experiments of Warburg revealed that the various metabolic quotients (aerobic and anaerobic glycolysis, etc.) of a wide variety of tumors were nearly the same from tumor to tumor (116). The Coris observed that the lactic acid and sugar contents of a sarcoma were very nearly the same as those of a carcinoma (117). In an analysis of the tissue metabolism data, Burk suggested that at least 95 per cent of all tumors fell within certain definite criteria (118). Williams and co-workers (119) observed that the content of a number of the B vitamins, and Robertson (120) observed that the content of ascorbic acid, in a variety of tumors, fell within relatively narrow limits. Salter *et al.* (121) and Burk and his colleagues (122) noted that the respiratory response of various malignant tissues to added substrates all fell within certain low limits. Shack (123) observed that the cytochrome oxidase activity of a number of mouse tumors was very nearly the same. The reviewer has determined the activity of a number of enzyme systems and the concentration of other chemical components in a wide variety of tissues and has noted (*a*) that the enzymatic pattern of a tumor is almost completely independent of its etiology, of its growth rate, and of the strain of animal in which it may be implanted, and (*b*) that all tumors, irrespective of their origin or site, produce identical systemic effects on their host (115). In contrast with the results on the tumors noted above, the investigators cited observed wide variations among normal tissues for the different components described. As an example, the enzymatic patterns of the normal gastric mucosa and of the normal liver are qualitatively and quantitatively vastly different, but those of the gastric adenocarcinoma (induced by methylcholanthrene) and of the hepatoma (spontaneous, or induced by aminoazotoluene or by carbon tetrachloride) are very nearly the same (115). Metabolically, tumors resemble each other more than they do normal tissues, or than normal tissues resemble each

other (124). The fact that certain properties of certain normal tissues overlap those of tumors (125 to 128) in no way invalidates the concept of a uniform metabolic behavior of tumors as a class. The conclusion may be therefore tentatively advanced that all tumors, no matter how or where they originate, tend toward a common tissue type.

Comparison of the activity of individual enzyme systems in homologous pairs of tumors and of their normal tissues of origin has revealed that systems of high activity in normal tissues, i.e., gastric pepsin, hepatic arginase and catalase, intestinal phosphatase, etc., are almost invariably drastically reduced in activity when the tissues become neoplastic (115, 129). The malignant transformation involves, therefore, a metabolic de-differentiation, or a loss in specific function. The tumor, although it has apparently lost most of the unique character of the tissue from which it arose, does not lose it entirely in every case. In certain respects, the hepatoma (130, 131) and osteogenic sarcoma (132, 133) bear some imprint of the tissue of origin and in some tumors of the thyroid and of the pancreatic islet cells hormonal secretions have been observed (134). Tumors, and least of all benign tumors, are not *per se* an identical tissue type; they are merely far more uniform in all respects studied than are normal tissues and apparently converge toward a uniform tissue type. The more malignant they are, the nearer they approach the kind of de-differentiated, functionless tissue presumably common to all neoplasms.

Two characteristic properties of tumors are (a) the fact that they grow, and (b) the evidence that they lack a marked differentiation in function. These are also properties of fetal tissue. Comparative studies of rat fetal liver, adult liver, and hepatoma by Burk (118) and by the reviewer (115, 135) have revealed, respectively, that the extent of glycolysis and the activity of a number of individual enzyme systems together with the sulfur distribution are all nearly the same in fetal liver and hepatoma and quite different from these tissues in the adult liver. Fetal liver possesses a certain enzyme pattern and sulfur distribution both of which alter to a quite different pattern and distribution in the adult liver; when however the adult liver becomes neoplastic, the adult pattern and distribution revert to an enzyme pattern and a sulfur distribution characteristic of those of fetal liver. Similar findings on biotin distribution have also been observed (93). The idea that tumors resemble or are analogous to fetal tissue goes far back to the earliest theories of cancer, but these are the first experimental data on the subject. Regenerating adult liver (after partial

hepatectomy) is a rapidly growing tissue, but it is fully differentiated metabolically (115, 118); functional activity is thus not incompatible with growth.

Attempts to correlate the respiration (123, 128) and the fermentation (136) of tumors with the content, respectively, of cytochrome-*c* and of zymohexase have revealed that in contrast with normal tissues tumors possess little if any reserve of these components (cf. 121). The metabolic functions of tumors appear to be conducted at the bare minimum level; like fetal tissues, tumors appear to possess a primitive economy.

Despite the fact that in a few properties there is an overlapping of some normal tissues and tumors, it may be accepted that normal tissues as a class and malignant tissues as a class are metabolically fairly well distinct (115, 118). Benign growths on the other hand, as might be expected, fall between these categories and may possess certain characteristics of normal and certain characteristics of malignant tissues (122, 128).

The widest range of benign growths is afforded by the clinic. In a study of the cytochrome-*c* content of a variety of human tissues, including normal organs and benign and malignant growths, it was observed that those normal tissues with a relatively low incidence of cancer (heart, liver, brain, kidney, muscle) possessed the highest concentration of this component, frankly malignant tissues possessed the lowest concentration, while those normal tissues with a relatively high incidence of cancer (lung, stomach, bladder, spleen, uterus) together with benign growths possessed a range of cytochrome-*c* concentration between those of the above extremes (128). In all tissues studied, in comparison with the concentration of cytochrome-*c*, cytochrome oxidase is present in excess. This excess is least in the case of the group of normal tissues which include heart, etc., greatest in the case of the malignant tissues, and intermediate between these extremes in the cases of those normal tissues which include lung, etc., and of benign growths. Similar findings may be drawn from the available data on rat tissues (115, 128, 137, 138).

*Properties of tumor-bearing animals.*—The presence of a tumor may evoke systemic reactions in a site far removed from that of the tumor. The unhappily familiar symptoms of clinical cachexia are an example. Rhoads, Abels, Pack, and their collaborators observed severe hepatic dysfunction in patients with gastrointestinal cancer which largely reverted to normal following operation (139). In laboratory

animals bearing tumors, the red cells and hemoglobin decrease (140), the blood proteose increases (141), the plasma zymohexase increases (136), and the serum and tissue esterase decreases (142, 143). These factors are restored to the normal level following regression or removal of the tumor. Of a large number of enzymes studied in several tissues, the reviewer observed that (a) catalase activity was considerably lowered in the liver and kidneys of animals bearing various kinds of tumors, (b) the extent of this lowering was proportional to the growth rate of the tumor, and (c) the catalase activity was restored to normal following operative removal or spontaneous regression of the tumor (115, 144, 145). Similar findings have been noted in the case of hepatic *d*-amino acid oxidase (146, 147). It is apparent that tumors of all kinds can effect a variety of systemic changes in the animals which bear them, and that such changes are reversible on removal of the tumor. The mechanism of these effects, which occur without any visible morphologic alteration of the affected tissues, is however yet obscure, nor is it definitely ascertained so far whether all of the effects noted are unique for tumors (148). Nearly all of these effects moreover are detectable only when the tumor weight is an appreciable fraction of the body weight of the host, thus rendering their practical clinical application at the present time rather remote. These effects however pose many fundamental problems, among them the puzzling question of why only certain enzymes in the liver or in the serum of the cancerous host are affected while others remain unaffected. The lowering of the liver catalase activity in tumor-bearing animals is not merely due to the presence of growing tissue *per se* in these animals, because the livers of pregnant mice or of mice bearing implants of minced, growing, embryonic tissue do not show this effect (149). Similarly, the presence of rapidly growing epithelial tissue in otherwise normal animals produces no effect on the hepatic *d*-amino acid oxidase (147). The low hemoglobin and catalase in cancerous animals suggest some interference with the mechanism for hematin synthesis. In any event, the fact that all kinds of tumors produce the systemic effects noted suggests that tumors possess properties in common and is consistent with the viewpoint stated above that all kinds of tumors tend to converge toward a common type.

In contrast with the obscurely caused effects described, which are apparently produced by all kinds of tumors, are the specific effects of the raising of the serum alkaline phosphatase activity in clinical cases of osteogenic sarcoma (133, 150) and of disseminated prostatic carci-

noma (151), and of the simultaneous raising of the serum acid phosphatase activity during the course of the latter disease (100, 103) (*vide infra*). The increase of these enzymes in the serum, obviously derived from the specific tissues involved, must be due to "leakage" from the tumor into the blood stream. Nondisseminated carcinoma of the prostate does not yield this effect (104). The involved tissues, bone and prostate, are so rich in these enzymes that only very little need escape into the serum to be readily picked by analysis and be used as either a diagnostic or prognostic test. For this reason, serum phosphatase tests are now routine clinical procedures in known or suspected cases of bone and prostatic disease. Operative removal of the osteogenic sarcoma results in a drop of the serum alkaline phosphatase to the normal level, followed by a rise as the tumor recurs (150). The reversibility of the serum acid phosphatase level following castration or estrogen administration in cases of disseminated prostatic carcinoma has been alluded to above.

Many of the symptoms of late neoplastic disease resemble certain features of adrenal cortical insufficiency. Two possibly related observations are (a) the severe loss of fatty material from the adrenals of tumor-bearing hosts (152, 153), and (b) the appearance in the urine of cancer patients of a number of highly oxygenated ketosteroids, not encountered in the urine of normal individuals, and suggestive of metabolites of the adrenal cortical hormones (154).

It is apparent that at the present time each approach has illuminated the problem of malignancy without as yet penetrating to its core. We know that a wide variety of agents will produce a tumor and that the tumor once formed is metabolically very much like all other tumors. The core of the problem lies in the nature of the malignant transformation. So far we know reasonably well only states A and Z, i.e., the fully normal and the frankly malignant. Recognition of such states as H, M, and P, as exemplified among others by the valuable series of studies by Cowdry's group on epidermal carcinogenesis (cf. 155), should be one of the future aims of investigation. These intermediate states occur in profusion in the clinic, and further progress in this field must involve a closer degree of collaboration between laboratory and clinic than has—regrettably—been the case up to now.

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## NITROGENOUS CONSTITUENTS OF PLANTS

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During the nine years since this subject was last reviewed in *Annual Review of Biochemistry* (1) four review articles dealing with various phases of protein metabolism in plants have appeared (2 to 5). The last named (5), concerned mainly with the nature of protein synthesis, is especially valuable.

At the present time the concept of protein metabolism in plants is closely analogous to that held for protein metabolism in animals. The amino acid composition of leaf proteins of angiosperms is now fairly well known through the work of Lugg, Tristram & Chibnall and has been summarised by the latter (4). The partial analyses of tissue proteins of a liverwort, a lycopod, and a fern by Lugg (6) indicate that the proteins of assimilating tissue do not differ widely in their amino acid patterns throughout the plant kingdom. In this respect they appear to be analogous to the muscle proteins of animals (7). Distinctions between "endogenous" and "exogenous" metabolism of protein have never been made by plant physiologists. This viewpoint has been substantiated by Vickery, Pucher, Schoenheimer & Rittenberg (8) who have shown that, when nitrogen of atomic weight 15 is administered as ammonium chloride to rapidly growing plants, the labelled nitrogen is rapidly assimilated into nitrogen of amides, amino acids, and proteins. Their findings provide evidence that continuous chemical interaction occurs between the nitrogen of tissue constituents and of supplied nutrients. Borsook & Dubnoff (9) have pointed out that the concentrations of proteins, amino acids, and ammonia in tissues is far from the thermodynamic equilibrium state; and the "steady state" in tissues is a dynamic state maintained by constant input of chemical potential energy through coupling with oxidative reactions.

Whether explicitly stated or not the ideas expressed in the last paragraph have been implied in most of the work on protein metabolism in plants in the period under review. The major advance in this subject since 1936 has been recognition of the interactions which exist between protein metabolism and respiration rate and organic acid metabolism. Various workers have sought for significant relations

between different metabolites and have endeavoured to formulate the probable course of protein metabolism in leaves.

The present review is restricted to work, which has appeared since 1936, dealing with (a) protein regulation in plants, and (b) precursors of amides and amino acids in plants. Concerning protein regulation in plants, two questions have been posed: Is the protein level in the leaf controlled by factors external to the leaf or by internal leaf factors? The first alternative implies hormonal control of some kind; if the second alternative is operative what is its nature? For the latter it has been suggested that protein regulation is determined by (a) mass action between ammonia and carbohydrates as initial reactants, (b) relations between proteins and amino acids, (c) the tempo of respiration. The search for precursors of amides and amino acids has led to determination of intermediate compounds which might link protein and carbohydrate metabolism. The above questions are discussed separately in this review.

*The question of hormonal control of protein synthesis.*—One factor which determines the protein level in mature leaves is the formation of new leaves or of inflorescence in which protein synthesis is active and which act as sinks and increase the rate of translocation of soluble nitrogenous compounds from mature leaves (10, 11). Other factors may be superimposed upon this and Chibnall (4), influenced by the fact that in detached leaves protein hydrolysis can be detected within a few hours after severance, suggested that "some influence of the root system, possibly hormonal, is responsible for the regulation of the protein level in leaves." The reviewer has seen in abstract only the claim of Kursanov & Brjuškova (12) that in wheat leaves the property of synthesizing protein is lost simultaneously in all leaves during formation of the inflorescence but is resumed in all leaves during flowering. It has also been suggested by Mothes (13) and McCalla (14) that leaves, even when attached to the plant, completely lose their property of protein synthesis at maturity.

These suggestions are subject to experimental verification. Walkley (11) and Walkley & Petrie (15) have shown that in decapitated barley plants from which tillers and all leaves above the fourth leaf had been removed, the mature fourth leaf, attached to the plant, can synthesize protein, although the capacity for synthesis decreases with age. These observations whilst disposing of the theory of the inability of mature leaves to synthesize protein might still be regarded as evidence for the hormone theory. Pearsall & Billimoria (16, 17) floated

leaves of several dicotyledonous plants and of *Narcissus* on nutrient solutions and, under the conditions of their experiments, found that mature leaves appeared to be incapable of synthesizing protein, although the basal elongating portion of *Narcissus* leaves possessed this property. On the other hand, Phillis & Mason (18) have shown that discs punched from mature cotton leaves could form protein as readily as intact attached leaves, although they found that with increasing age the leaf required a higher level of "crystalloid"-nitrogen to maintain a definite protein level. Stewart *et al.* (19) have recently shown that in potato tubers stored at 2° C. the amount of protein decreases and amount of amino nitrogen increases with time of storage; with increasing age, discs of the tubers lose their power of synthesizing protein when transferred to aerated solutions. On the other hand potatoes stored at 11° C. do not lose protein, amino-nitrogen content remains constant, and the power of protein synthesis is not lost with increasing age. The authors ascribe the loss of synthetic property at low temperatures to loss of oxidase activity by the tissue.

The data of different observers on different species are at variance but it is clear that in some plants at least, the property of protein synthesis is not lost in mature leaves, whether these are attached to or detached from the plant, and in these cases the view that synthesis of protein is determined by factors external to the leaf cannot be maintained; however, it is clear that in attached leaves the presence of sinks may cause increased rate of translocation of soluble nitrogen compounds. It is also clear that the capacity of the leaves for synthesis decreases with the age of the leaf; the cause of this remains speculative, but is possibly connected with changes in enzyme systems concerned.

*Relations between proteins and carbohydrates.*—Paech (20) suggested that amount of protein is controlled by mass action, the reactants being ammonia and carbohydrates (monoses). This hypothesis was based chiefly upon work with starving leaves in which decrease in amount of protein is associated with decrease in amount of carbohydrate, although Dastur, Kanitka & Rao (21) have suggested (without analytical data) that in light, rate of protein synthesis is dependent on rate of carbohydrate production. Chibnall (4) has reviewed work on which Paech's hypothesis was based and has criticised the hypothesis, pointing out the weakness of Paech's assumption that the active mass of monoses determines the active mass of  $\alpha$ -ketonic acids, the probable precursors of amino acids. He pointed out however that in

the data cited by Paech, although amounts of protein were estimated, corresponding analyses of carbohydrates were not available.

Kabos (22) found that protein increased in amount in mustard seedlings grown in complete culture solutions containing up to 2 per cent sucrose, but that further increase in sucrose concentration up to 6 per cent caused no further increase in amount of protein; sucrose was more effective than glucose in maintaining a given protein level.

Amos & Wood (23) supplied rye grass with varying amounts of ammonium salts and analysed the leaves after allowing the plants to approach a steady state in a constant (illuminated) environment. They found that sucrose, glucose, fructosans, hemicellulose, and starch, but apparently not fructopyranose, were utilised in formation of protein, but that under the conditions of their experiment increase in amount of protein was associated with decrease in amount of each carbohydrate. Wood & Petrie (24) described an experiment designed to test Paech's hypothesis and also the question of the path of protein synthesis. They pointed out that if protein is formed from a special set of amino acids as suggested by Block (25), or not from amino acids but along another path, then protein could, in practice, be related to the ultimate reactants viz. ammonia and carbohydrate. Using the constant environment technique described below, Wood & Petrie applied differential treatments of sucrose and ammonium salt to *Phalaris* plants. Like Amos & Wood, they found a high negative correlation of protein on sucrose and glucose contents, whilst the relation on fructose was insignificant; nor could they find a significant correlation of protein on any function of the ammonia content. On the other hand they found that the variance of protein content could be almost wholly accounted for in terms of the amino acid and water contents, all expressed on a dry weight basis. They concluded that Paech's hypothesis was untenable and also that proteins were probably formed from the whole of the amino acids rather than along any alternative path. Further, it was clear from their data that carbohydrate content did not affect the relations between protein and amino acids; nor did carbohydrates, acting through respiration rate, affect these relations, for respiration rate showed a high negative correlation with contents of the different carbohydrates.

Wood, Cruickshank & Kuchel (26) describe several experiments with starving detached leaves of Sudan grass and Kikuyu grass. Generally the leaves showed immediate proteolysis on being placed in darkness similar to that described by Yemm & Vickery, whose work

has been summarised by Chibnall (4), and there was a formal resemblance between the curves plotted for protein vs. time and sucrose vs. time, though not for hexoses vs. time. In one experiment, however, the plants were illuminated in a constant environment cabinet for two days before cutting; under these conditions the sucrose content rose to a very high level. On starvation the sucrose content fell rapidly but the protein content and also the amino acid content remained approximately constant over a period of three days. Vickery & Pucher (27) also describe an experiment with rhubarb leaves, cultured in darkness, in which no change in amount of protein occurred during the first twenty-four hours of starvation: in these leaves carbohydrate content was high. In leaves in which carbohydrate content was low proteolysis commenced immediately after darkening the leaves. These findings are similar to those reported by Deleano (28) for vine leaves. Wood & Petrie (24) point out that in all experiments with the constant environment cabinets the sucrose content was high and that the relation between furanose and  $\alpha$ -ketonic acids (the probable precursors of amino acids) is probably of the Michaelis type, and similar to the relations between respiration rate and sucrose which Barker (29) has shown to be of the Michaelis form. It might be expected that in the starvation experiments in which protein and carbohydrate contents decrease simultaneously the carbohydrate content probably limits amino acid formation; when carbohydrate concentration is high the amino acid and protein contents would be independent of the carbohydrate concentration, though not necessarily independent of the rate at which carbohydrate passes through an oxidative cycle.

The experiments described above present a coherent and plausible basis for such a hypothesis but certain experiments have been described which suggest that complications occur in leaves in the light. Vickery *et al.* (30) found that in detached leaves of tobacco, cultured in the light, loss of protein occurred although the carbohydrates increased in amount; loss of protein was less in the light than in the dark, however. Furthermore, they found that glutamine increased in amount in the light but was not formed in darkness. They were unable to explain these data but referred to possible complexities of reactions in detached leaves.

It should be pointed out here that in the experiments of the writer and his collaborators ammonium salts have always been used in order to overcome possible complications concerned with nitrate reduction. In this connection the work of Pearsall & Billimoria (17) is of inter-



est. These investigators found that light *per se*, and apart from photosynthetic effects, caused increased absorption of nitrogen, increased nitrate reduction, and increased production of soluble organic nitrogen in detached leaves floating on nutrient solutions. Blackman & Templeman (31) found negative correlations of proteins on carbohydrates in grass leaves. These investigators grew grass plants in full daylight and with varying degrees of shading (0.6 to 0.4 full daylight). In full daylight they found gain of protein with both ammonium salts and nitrate used as a source of nitrogen but at lower light intensities they found that elaboration of protein was reduced and that nitrates accumulated. They concluded that with high nitrogen supply shaded leaves lose their ability to elaborate nitrate, rather than ammonia, into organic nitrogen. The ability to reduce nitrate was not dependent on the carbohydrate content.

Relations between proteins and carbohydrates cannot be fully discussed without reference to the effect of respiration rate on protein level and on relations between proteins and amino acids. Further consideration of effects of carbohydrates is therefore postponed until the following sections.

*Relations between proteins and amino acids.*—Before describing work on relationships between proteins and free amino acids in the cell sap it appears appropriate to describe the method employed in the writer's laboratory in investigating relations between metabolites. The method consists in submitting plants of uniform ontogenetic age to constant environmental conditions in a cabinet, both before and after application of experimental treatments. With such constancy of environment there is a better chance that the systems within the leaf approach a drifting steady state. In such a state changes in the factors defining the system are so slow that they result in a succession of states each of which is inappreciably removed from a steady state. Data were obtained over a period of from two to four days in order to minimize ontogenetic drifts and changes in dry weight; and the data were then subjected to statistical analysis. The concepts involved have been discussed by Petrie & Wood (32) and Petrie (5).

Using this method Petrie & Wood (32) in a series of experiments found a well marked relation to hold between concentrations of proteins and amino acids such that the curve relating them is concave to the amino acid axis. The same form of relationship held between protein and total amino acids and protein and residual amino acids (*i.e.*, total  $\alpha$ -amino nitrogen minus amino nitrogen of the amides asparagine

and glutamine). Wood & Petrie (24) and Wood (33) also found the relation to hold in an experiment in which carbohydrate content varied widely but in which low water content was not always associated with high amino acid content as in the experiments previously described.

The relations observed by Petrie & Wood occurred in leaves during a brief period of time in which synthesis of protein occurred. Walkley & Petrie (15) investigated relations between proteins and amino acids during longer periods of time and at different stages of the life cycle of grass plants, using the mature fifth leaf of decapitated barley plants, in a constant environment with and without the addition of ammonium salts to the soil in which the plants were growing. They found that during the phases of both protein synthesis and protein hydrolysis the relations between concentrations of proteins and amino acids could be expressed by a curve concave to the amino acid axis. The main factor causing protein hydrolysis was removal of amino acids to other sinks. They also obtained some evidence for a time drift in the protein-amino acid relationship such that with increasing age of the leaf the amount of protein associated with a given value of amino acid becomes progressively smaller.

The work of Richards & Templeman (69) suggests that potassium deficiency may affect the position of balance between proteins and amino acids; and also, that phosphorus deficiency increases the ratio of amide to amino acid nitrogen. As previously pointed out by Petrie (5) their data suffer from the disadvantage of being obtained from structurally nonuniform material.

Phillis & Mason (18, 67) have reported a relationship between protein and "crystalloid" nitrogen (i.e., sap-soluble nitrogen) similar to that described above between protein and amino acid nitrogen to hold throughout the life cycle of leaves of cotton. Although the "crystalloid" nitrogen contains a variety of organic as well as inorganic nitrogen compounds and cannot have the value of distinct chemical entities, this work may be regarded as confirming that described above. Phillis & Mason also describe an age effect such that with increasing age the leaf requires an increased "crystalloid" nitrogen concentration to produce a standard protein level.

Phillis & Mason have ascribed the relations found between proteins and amino acids to apolar adsorption of the type described by Buzagh (34) and have suggested that "crystalloid" nitrogen is adsorbed before conversion into protein. This view, as well as the statis-

tical methods of the authors, has been severely criticised by Richards (68).

The writer of this review and his collaborators think that the form of the relations between protein and total amino acid concentrations suggests that some other factor is limiting. Petrie & Wood (32) pointed out that any relationship between proteins and total amino acids holds only so long as the proportions among the free individual amino acids remain constant and suggested that at least one factor determining the concavity of the protein-amino acid curve could be explained if certain amino acids were oxidised more rapidly than others as the total amino acid concentration increased. They showed (32) that the concentration of cystine did not increase when the concentration of other amino acids increased. Wood (33) investigated interrelations between respiration rate, protein, and amino acids and concluded that these could best be explained by the hypothesis that respiration rate increased as the amino acid concentration increased and that some amino acids were more readily deaminated than others. The observation of Lugg & Weller (35) that the amount of protein in legume seedlings appears to be limited by the amount of methionine present in the seed appears to be an application of this principle.

The main course of nitrogen metabolism in leaves starved in darkness has been investigated in barley (36), tobacco and rhubarb (30, 37), Kikuyu grass, Sudan Grass and oats (26, 38). Except when carbohydrate content is very high, the series of events which takes place under these conditions is the same in all cases, viz., protein hydrolysis commences immediately on placing the leaves in darkness, and the protein content decreases in an approximately linear fashion with time until chloroplast disintegration occurs. Wood, Cruickshank & Kuchel (26) isolated and determined the amount of chloroplast protein and showed that this decreased at approximately the same rate as cytoplasmic protein during starvation. During this period the amounts of residual amino acids and amides successively attain maximum values and then decrease. Ammonia increases throughout the starvation time and reaches a maximum later than does asparagine. The implied oxidation of amino acids to ammonia and carbon residues and subsequent recombination in part to form amides is well known.

Wood & Cruickshank (39) have shown that in Kikuyu grass and in oats starved in air there is a preferential utilization of amino acids. They determined the amounts of some amino acids, free in the cell

sap, which could be estimated readily in small amounts, and found that in all cases the order of utilization of those estimated was cystine, glutamine, arginine, tyrosine, and tryptophane. They found the content of cystine and of glutamine to reach a maximum before the end of the second day of starvation, the cystine maximum occurring earlier than that of glutamine; the arginine maximum occurred at about the end of the second day. The amounts of these three acids decreased after reaching a maximum; and in all cases the amount of each present was less than that expected from the amount of protein hydrolyzed. The amount of tyrosine attained a maximum between the second and third days and tryptophane between the third and fourth days of starvation; thereafter, both decreased in amount. During the first two or three days of starvation these two acids were not readily transformed since the amounts present corresponded with those expected from the amount of protein hydrolyzed. In contrast to the effects in air, Wood & Cruickshank found that there was little change in amounts of protein and amino acids when leaves were starved in nitrogen unless injury occurred. Under the latter circumstances proteins decreased in amount and amino acids accumulated in an equivalent amount but no secondary formation of amides occurred. Wood & Cruickshank concluded that a relationship between proteins and total amino acids may occur in starving leaves similar to that found in leaves in which synthesis of protein occurs, i.e., a reversible relation may exist between proteins and the whole of the amino acids but modified by preferential oxidation of some amino acids. This oxidation leads to a disproportionality among the amino acids so that protein hydrolysis occurs. Therefore, a given protein content may be in equilibrium with a varying total amino acid content.

Gregory & Sen (40) dismissed the possibility of a mass action regulation between proteins and amino acids on the ground that the ratio of protein to total amino acids did not tend towards a constant value. They put forward the hypothesis that protein synthesis and protein hydrolysis are two separate processes and catalysed by separate enzyme systems. Such a hypothesis seems unnecessary in the light of the work just described.

*Relations between proteins, amino acids, and respiration rate.*—Several workers have sought for correlations between proteins and respiration rate, with the underlying idea that the latter might give a measure of amount of energy expended in maintaining protein content.

Gregory & Sen (40) grew barley plants at various levels of potassium and nitrogen deficiency with phosphorus content held constant and in excess. They found that in nitrogen deficiency respiration rate was correlated with amino acid and with protein contents, the two latter variables themselves being positively correlated; in potassium deficiency respiration rate was correlated with protein and carbohydrate contents. In the nitrogen-deficient plants carbohydrate content was high but in potassium-deficient plants it was low. Richards (41), using the same technique, investigated the effects of phosphorus deficiency. He found that in normally manured plants and in cases of mild phosphorus deficiency respiration rate was correlated with protein and amino acid contents, the two latter themselves being correlated; and in extreme phosphorus deficiency respiration rate was correlated with protein and with carbohydrate contents. In the former case carbohydrate content was high and in the latter case relatively low.

It is noteworthy in these experiments that carbohydrate content was high in cases where correlations exist between respiration rate and amino acid content. Barker (29) has shown in potatoes that the relation between respiration rate and carbohydrate content can be expressed by a hyperbolic function as expected from the Michaelis equation, so that at high carbohydrate concentrations respiration rate is independent of carbohydrate content. Wood (33) in one experiment increased the carbohydrate content of grass leaves by adding sucrose solutions to pots in which relatively nitrogen-deficient plants, already high in carbohydrates, were growing. Under these circumstances amino acid and protein contents remained practically unchanged but carbohydrate content reached high values. Respiration rate under these conditions remained approximately constant and independent of carbohydrate content. Lack of correlation between respiration rate and carbohydrate content does not therefore imply lack of real effect but simply that the effect may be approaching its maximal limit. When carbohydrate content is high, therefore, the relationship between respiration rate and amino acids becomes more marked.

Petrie & Williams (42), and also Wood (43), found correlations between respiration rate and protein content in leaves analysed at various stages of the life cycle of grass plants and concluded that these correlations might occur because protein might be a measure of the number of respiratory seats, fluctuations in rate being accounted for in terms of various amino acid or sucrose contents.

Richards (41), struck by the constancy of respiration rate expressed on a protein basis in his experiments on phosphorus-deficient plants, concluded that there exists a reciprocal relationship between proteins and amino acids so that a given rate of carbon dioxide evolution can maintain only a definite quantity of protein.

Steward & Preston (44, 45) and Steward, Stout & Preston (46) studied discs of potato tuber immersed in aerated solutions of chlorides, bromides, and nitrates of potassium and calcium and of potassium sulphate, and concluded that the rate of protein synthesis depended on the rate of carbon dioxide production. Their work has been criticised by Petrie (5) on the ground that it is unwise to attach too much significance to relations between respiration rate and protein content in a system in which cell division is actively occurring. More recently Steward *et al.* (19) have implied that only a part of the carbon dioxide production is linked with processes concerned in protein synthesis.

Petrie & Wood (32) and Wood & Petrie (47) supplied ammonium salts to plants and caused considerable changes in amounts of amino acids in the leaves; they found that respiration rate showed high correlations with amino acid content. Wood & Petrie (47) and Wood (33) showed that during protein synthesis the amount of protein was determined primarily by amino acid concentration. They found that with reduction of water content amino acid content and respiration rate increased whilst protein content decreased. They concluded from their data that the most likely explanation was that with increased amino acid concentration some amino acids were deaminated more rapidly than others with consequent changes in the proportions among the amino acids and this resulted in protein hydrolysis.

It is apparent from the foregoing observations that carbon dioxide evolution depends upon oxidation of other substances than carbohydrates during synthesis of protein in the light. This is also true of leaves starved in air in darkness.

Yemm (36) showed that in starved leaves of barley total carbohydrate loss accounted for a large fraction of the carbon dioxide evolved during the first few hours of starvation, but that thereafter the bulk of the carbon dioxide evolved was derived from sources other than carbohydrate; the value of the respiratory quotient suggested that the latter were amino acids. Vickery & Pucher (27) determined the distribution of total and soluble carbon at various times during the starvation of rhubarb leaves; they determined also amounts of

protein, total amino acids, amides, carbohydrates, and organic acids and showed that respiration draws upon components other than carbohydrates and suggested that these were amino acid residues. As pointed out in the previous section it has been shown by Yemm (36), Vickery *et al.* (30, 37), and Wood *et al.* (26, 38) that in starving leaves there occurs a progressive loss of protein; with increasing time of starvation residual amino acids and amides successively attain maximum contents and thereafter decrease in amount; ammonia increases in amount throughout the starvation time. From the changes in amounts of these nitrogen compounds, and also in amounts of carbohydrates, between successive time intervals, Wood, Mercer & Pedlow (38) have calculated the amounts of carbon dioxide evolved from oxidation of carbohydrates on the one hand and by transformations of nitrogen compounds on the other, and compared these with the value of carbon dioxide evolution actually obtained. Yemm (36) and Wood *et al.* (26, 38) have shown that although protein content decreases in amount during starvation; yet respiration rate shows no correlation with protein content and during the climacteric period rises whilst protein content decreases.

Wood & Cruickshank (39) point out that whilst the view, that a given rate of carbon dioxide evolution can only maintain a definite quantity of protein, cannot be substantiated in all cases, the data of all observers are in harmony with the hypothesis that protein content in leaves may be determined by the rate at which carbohydrates are passing through an oxidative cycle. They point out that all their work suggests that proteins and amino acids exhibit relations which can be interpreted in terms of mass law, modified by the fact that some amino acids are oxidised more readily than others. They consider it a reasonable assumption that a definite level of protein can be maintained in leaves in air, provided that synthesis of the most readily oxidised amino acids takes place at the same rate as their oxidation. Such a state of affairs would depend in the last analysis on the rate of breakdown of carbohydrates which provides intermediary substances for amino acid formation. They point out that in experiments with starved leaves in which carbohydrate content is initially high (26, 27, 28) no change in amounts of proteins or amino acids occurs until after many hours; thereafter, protein decreases in amount and this decrease is correlated closely with decrease in amount of soluble carbohydrate, though not with rate of carbon dioxide evolution.

The hypothesis outlined in the last paragraph appears to account satisfactorily for relations between proteins, amino acids, carbohydrates, and respiration rate when leaves are starved in air but is not sufficient to account for their behavior when starved in an atmosphere of nitrogen. Wood *et al.* (26, 38, 39) have shown that when leaves are starved in nitrogen loss of carbohydrates occurs as rapidly as in air and respiration rate falls to a low level; protein content however decreases only very slightly in amount, except in cases of injury, and amino acids increase slightly in amount but no asparagine is formed.

It appears, therefore, that in nitrogen proteolytic enzymes in some way are prevented from coming in contact with their substrates. Linderstrøm-Lang (48) has pointed out that many native proteins are not hydrolyzed by enzymes until denatured and has suggested that this has its origin in steric hindrance, especially in blockage or combination of peptide groups within the molecule, and that in protein hydrolysis an initial reaction takes place prior to fission of the peptide bonds. Wood & Cruickshank (39) have suggested that steric hindrance in a wider sense than this might retard protein hydrolysis. Wood *et al.* (26, 38) have determined the amounts of chloroplast protein, chlorophyll, and ascorbic acid in leaves during starvation both in air and in nitrogen. They showed that the amount of each of these substances remains approximately constant over long periods in leaves starved in nitrogen. When leaves are starved in air, however, chloroplast protein, chlorophyll, and ascorbic acid all decrease in amount at approximately the same rate, but if the initial carbohydrate content is very high then breakdown of all these substances is retarded. Recent work (49) has focussed attention on the existence of a pattern within the chloroplast in which protein, chlorophyll, carotenoids, ascorbic acid, and phosphatides are all interlocked components. Strain (50) has shown that xanthophylls are not readily oxidised unless the chloroplast structure is destroyed. Smith & Robb (51) have pointed out the close correlation between nitrogen and  $\beta$ -carotene contents in the oat plant. Moyer & Fishman (52) have prepared chlorophyll-protein complexes from plants and determined their electric mobility curves. They conclude that the complex from each species is characterised by essentially the same protein.

Wood & Cruickshank (39) have suggested that it is the existence of this pattern which protects the protein from hydrolysis in an atmosphere of nitrogen. Similar patterns, but without pigments, probably occur in the cytoplasm for Wood *et al.* (26) have shown that



chloroplast protein and cytoplasmic protein both decrease at approximately the same rate during starvation. Wood & Cruickshank (39) have pictured this pattern as static in an atmosphere of nitrogen, not subject to oxidation of the components and the protein protected from enzymes by its existence. In air, on the other hand, all components are subject to oxidation and the static pattern is replaced by a dynamic one in which each component undergoes synthesis and degradation. On temporary breaking of the pattern during oxidation, enzymes may come into contact with their substrates. Whether or not the components of the pattern remain present in any given amount will depend upon whether their rates of synthesis are equal to their rates of oxidation; and that this in turn will be determined in large measure by the rate at which carbohydrates traverse a respiratory cycle.

*Formation of amino acids and amides.*—A great deal of work during the period under review has been concerned with the nature of the precursors of the amides, glutamine and asparagine, and with the way in which amides enter into the general scheme of protein metabolism. Review articles on amide metabolism in plants have been published by Vickery & Pucher (53), Vickery *et al.* (37) and Chibnall (4).

Schulze (54) provided the fundamental basis of amide metabolism from his work on seedlings in which he recognised that amides arise by the reaction of ammonia, derived from amino acids of the seed proteins, with nitrogen-free substances; and that they may be stored temporarily but may be drawn upon for regeneration of protein in growing regions. Schulze considered that the carbon compound combining with the ammonia had its origin from carbohydrates. Prianischnikow & Schulow (55) first suggested the idea, which gained much support, that the chief function of amides was to provide a means of removal of ammonia which might otherwise accumulate in quantity and prove toxic to the plant.

Wood & Petrie (47) and Wood (33) supplied different amounts of ammonium salts to plants of uniform ontogenetic age and determined amounts of various nitrogenous constituents after the plants had approached a steady state. They found that the curves relating residual amino acid content to ammonia content and amide content to ammonia content were concave to the ammonia axis; although not necessarily a logarithmic function, the relations between the two variables could be expressed simply and satisfactorily by regression equations in which amide or amino acid content was a function of the

logarithm of the ammonia content. Respiration rate and carbohydrate content were apparently not parameters in the relationship over the whole of the data. It was concluded that amides (and amino acids) increased in amount with increased ammonia content of the leaves to a point at which no further increases occurred even with large increases in ammonia content. At this point Wood & Petrie considered that some other factor, probably concentration of a carbon compound, was limiting. In experiments with plants supplied with ammonium salts in the light both glutamine and asparagine were highly correlated with residual amino acid content (33).

Vickery & Pucher (53) and Vickery *et al.* (56) grew tobacco plants in sand culture with solutions in which the relative proportions of nitrate nitrogen and ammonia nitrogen were varied, the total nitrogen content of all solutions remaining constant. They analysed their plants at the time of first flower bud formation and found that although the ammonia content increased to high concentrations in the leaves yet there was little increase in amide content with increased ammonia content. Soluble carbohydrates and organic acids decreased in amount with increasing ammonia content. They considered, therefore, that Prianischnikow's view that synthesis of amides results from an effort of the cells to maintain a low level of ammonia is inadequate to account for the behaviour of amides, but that the amount of amides was determined by the amounts of nitrogen-free precursors as well as by the amount of ammonia present.

Vickery *et al.* (30) point out that present day views regard the most probable precursor of an amino acid to be the corresponding  $\alpha$ -keto acid. In the cases of asparagine and glutamine, the problem narrows down to a search for a mechanism whereby oxalacetic and  $\alpha$ -ketoglutaric acids may be produced in plant tissues.

Gregory & Sen (40) established relationships between respiration rate and protein and carbohydrate metabolism which have been discussed in previous sections of this review. To account for these relationships they postulated that a protein cycle is continuously at work in leaves (they considered the paths of protein synthesis and degradation to be separate); the nitrogen-free residues of amino acids are respired; and amino acid synthesis takes place by way of organic acids derived from sugars.

The scheme of Gregory & Sen is a highly generalized one but has been given greater precision by Chibnall (4) who has suggested that the citric acid cycle of Krebs & Johnson (57) provides a chemical

mechanism whereby both oxalacetic and  $\alpha$ -ketoglutaric acids may be formed. These acids may react with ammonia to form aspartic and glutamic acids in the presence of aspartic or glutamic acid enzymes respectively, and react further with ammonia to produce amides; or amino acids may transfer amino nitrogen to the keto acids by "transamination" (58). Chibnall (4) showed that if the ammonium salt of  $\alpha$ -ketoglutaric acid is infiltrated into leaf tissue, this substance decreases in amount and a simultaneous increase in glutamine occurs. Chibnall's scheme has been treated exhaustively in his book (4) and also by Vickery *et al.* (37). Albaum & Cohen (59) have shown that transamination between glutamic and oxalacetic acid occurs in oat seedlings, the forward reaction proceeding at a rate three times as great as the reverse reaction.

Vickery *et al.* (30, 37, 60) have determined amounts of malic and citric acids as well as amounts of nitrogenous compounds and carbohydrates in starved leaves of tobacco and rhubarb; and Wood *et al.* (26, 38) have determined the same compounds and also respiration rate in starved leaves of Kikuyu grass and Sudan grass. Malic and citric acids behave differently during starvation in the different species investigated. They increase in amount with increasing starvation time in some species and decrease in amount in others, although in all cases the amount of amide increases. Vickery *et al.* (56) showed that supply of nitrogen as nitrate caused large amounts of both malic and citric acids to accumulate in plants, whilst when nitrogen was supplied as ammonium salts the amounts of these acids were very small; amount of soluble carbohydrate was also less in the ammonium-treated plants than in the nitrate-treated plants, but no simple proportionality existed between amounts of acids and carbohydrates. In both types of plants, however, the amounts of amides were approximately the same. If the Krebs-Johnson cycle is operative it would appear, therefore, that only a fraction of the total malic or citric acid exhibits relations with the amides. Unpublished work by the reviewer suggests that amount of amides is correlated with rate of carbohydrate oxidation rather than with organic acid content, when ammonia is in excess. Recent work by Wood *et al.* (61) and by Evans & Slotin (62) using isotopic carbon has suggested that *cis*-aconitic acid rather than citric acid is an intermediary between oxalacetic acid and  $\alpha$ -ketoglutaric acid, citric acid being formed as a side reaction. It is also probable that carboxylation of pyruvic acid by carbon dioxide to form oxalacetic acid occurs, since Ruben & Kamen (63) have shown that

unicellular algae assimilate carbon dioxide which becomes fixed in carboxyl groups in the dark. These observations suggest that the concentration of pyruvic acid may control the amount of  $\alpha$ -ketoglutaric acid and, in part, the amount of oxalacetic acid.

The transformation of malic acid into citric acid reported in tobacco leaves in darkness by Vickery (30) and its nonoperation in other leaves suggests differences in enzyme equipment in different species. Differences between species in ability to form amides in darkness are also apparent. For example leaves of tobacco (30) and Kikuyu grass (26, 38, 39) produce large quantities of asparagine, but apparently there is no secondary formation of glutamine in darkness; in barley (36) and oats (39) secondary synthesis of both glutamine and asparagine occur in darkness; in rhubarb (37) glutamine only but not asparagine is synthesized. Vickery *et al.* (30) have also found that no glutamine but only asparagine occurs in tobacco leaves in darkness, and yet both amides accumulate in leaves kept in the light. Vickery & Pucher (64) have shown that in etiolated seedlings of *Lupinus angustifolius* and *Vicia atropurpurea* asparagine with only negligible amounts of glutamine is formed, but in *Cucurbita pepo*, glutamine is formed with relatively little asparagine. Differences in enzyme equipment, in carbohydrate content, and possibly also in details of chemical mechanism in light and darkness probably form the basis of the observed differences in behaviour in different species.

The scheme formulated by Chibnall (4) and incorporating recent modifications of the citric acid cycle is a speculative one. As pointed out by Vickery (37), perhaps the strongest argument in its favour is the purely pragmatic one that for the present it permits a rational explanation of many relationships between well-known plant constituents and respiration. Desiderata for the future are more extensive analyses in different plant materials of substances entering into the general metabolic scheme of which protein metabolism is a part, and especially determinations of organic acids; for greater precision in attempts to separate cytoplasmic and vacuolar sap along the lines commenced by Mason & Phillis (65) and by Bennett-Clark & Bexon (66); and for detailed investigations into the nature, activities, and amounts of the enzyme systems concerned.

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## BIOLOGICAL NITROGEN FIXATION

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Because of the economic importance of the subject for agriculture, researches on biological nitrogen fixation are seldom lacking, but it is questionable how many of these possess interest for the chemist. Since the last review (1) a voluminous literature has again accumulated, and as before, the reviewers must exercise selection. Although the guidepost of biochemical importance serves well in most instances, elimination of certain papers may appear to be personal bias as others of similar nature are included. In general, it is believed that contributions which deal with the following aspects of biological nitrogen fixation have less interest for the biochemist than those chosen for discussion: taxonomy and classification of the organisms including serological studies on the bacteria; agronomic investigations such as strain variation among the root nodule bacteria, commercial inoculation of leguminous plants and seed treatment, cross-inoculation groupings, and mixed cropping; research more properly of interest to workers in soil and plant sciences, e.g., role of bacteriophage in symbiotic nitrogen fixation, association between the azotobacter and other microorganisms, distributions of nitrogen fixing organisms in the field, cytology of the nodule, plant composition as influenced by nitrogen nutrition.

### AGENTS OF BIOLOGICAL NITROGEN FIXATION

Uncritical acceptance of claims of nitrogen fixation by various biological agents lead to the conclusion that this is a phenomenon of wide occurrence. Wilson (2), however, called attention to possible sources of error in experiments designed to test agents for their ability to fix nitrogen. The chief errors result from limitations of the Kjeldahl nitrogen analysis and sampling variations in handling heterogeneous material initially rich in nitrogen. Since gasometric methods are not subject to such errors Hurwitz & Wilson (3) proposed that claims for fixation be accepted with reservation unless confirmed by a suitable gasometric analysis. They described a procedure which would be satisfactory for such tests demonstrating its accuracy and sensitivity in trials with *Azotobacter vinelandii*. Allison, Hoover & Minor (4) employed a Warburg respirometer with mercury as the manometric fluid



for testing nitrogen fixation by excised root nodules of legumes. No fixation was observed although Virtanen (5) using a gasometric measurement had reported fixation by excised nodules from peas.

All the technical difficulties with their resultant disputes should be eliminated in future studies if fixation is based on use of an isotopic method. For example, if molecular nitrogen enriched with  $N^{15}$  is supplied, fixation is evidenced by an increase of  $N^{15}$  over the normal concentration in the agent. Sampling errors are relatively unimportant, the objections leveled at the Kjeldahl analysis do not apply, and the isotope measurement is far more sensitive than gasometric, Kjeldahl, or other methods. Thus a method apparently is now available which should furnish an unequivocal answer regarding the ability of an agent to fix nitrogen. Exchange reactions between molecular nitrogen and fixed nitrogen compounds, however, would invalidate the results. A test (6) indicated that no exchange occurs in the presence of the azotobacter. The fact that numerous agents have given negative tests for fixation of  $N^{15}$  further substantiates the nonoccurrence of exchange reactions. However, Nishina and co-workers (7) reported exchange between molecular nitrogen enriched with  $N^{13}$  and a number of nitrogen compounds in solution. Repetition of these experiments by Norris, Ruben & Kamen (8) indicated that the exchange with sodium nitrate and nitrite was less than 0.01 per cent, much less than that reported by Nishina and co-workers. Using stable  $N^{15}$  rather than the radioactive  $N^{13}$ , Joris (9) also found no evidence of exchange.

Although inoculated clover plants utilized molecular  $N^{15}$  readily, barley plants and uninoculated clover plants furnished the same atmosphere fixed none (10). The experiments, covering periods of forty-two and fifty-six days, did not confirm the earlier report by Ruben and co-workers (11) which suggested that barley tops exposed to radioactive  $N^{13}$  for twenty minutes fixed molecular nitrogen.

Further application of the tracer technique showed that, in addition to the inoculated legumes, other well recognized biological nitrogen fixing agents, such as the free-living aerobic azotobacter, the blue-green alga *Nostoc muscorum*, and the anaerobic bacterium *Clostridium pasteurianum*, fixed large and readily determined quantities of molecular  $N^{15}$  (12). Negative results were obtained with several questionable agents tested, viz., germinating pea seeds, free-living root nodule bacteria, and cell-free preparations of the azotobacter. Excised nodulated legume roots consistently fixed small but readily detected quantities of  $N^{15}$ , but the excised legume nodules alone showed variable be-

havior. Fixation was evident at times but it was not regularly correlated with the addition of oxalacetic acid as has been claimed (5). Since symbiotic fixation was not ruled out in the experiments with excised nodules and nodulated roots, this factor may have influenced the irregular results observed. In other studies with isotopes (13) repetition of the oft-cited experiments of Golding (14) showed no nitrogen fixation by ground pea plants or by an extract of the pea inoculated with *Rhizobium leguminosarum*.

The role of algae in nitrogen fixation has recently attracted much attention following independent demonstrations by Allison and De and their co-workers (15, 16) that certain blue-green algae, grown under thoroughly controlled conditions, can fix considerable quantities of nitrogen. As mentioned (12), these observations have been confirmed using  $N^{15}$ . Stokes (17) showed that the suggested symbiosis between blue-green algae and the azotobacter could account for very little nitrogen fixation, since the meager quantity of carbohydrate supplied by the algae would support only slight growth of the azotobacter. Fogg (18) reported the failure of a nitrogen-fixing, blue-green alga to use molecular nitrogen in the presence of nitrate or ammonia. Bortels (19) has shown that, in common with the azotobacter, blue-green algae require molybdenum for active nitrogen fixation. The similarity of the nitrogen fixing mechanism of algae, the azotobacter, and nodulated leguminous plants is further emphasized by their common sensitivity to hydrogen and to carbon monoxide (20).

Dhar and co-workers (21, 22) attribute a considerable portion of nitrogen fixation in soil to photochemical reduction of nitrogen. They contend that the addition of carbohydrate to sterile soil is accompanied by a disappearance of the carbohydrate and the fixation of nitrogen, the process occurring in the dark but being accelerated by light. Sulaiman (23), also working in India, reported a decrease in sucrose in soil samples accompanied by an increase in percentage nitrogen, the increase being greater in light. There was no attempt to control bacterial fixation and the maximum fixation, a shift from 48 mg. nitrogen per 100 gm. dry soil to 48.8 mg. in the dark and 49.2 mg. in the light, is scarcely an impressive change. Volz (24), in an experiment carefully designed to check alleged claims of fixation of nitrogen by nonbiological decomposition of carbohydrate, found no significant evidence that such reactions occur. When transferred to an acre basis Dhar's results apparently indicate considerable fixation; when the data for individual samples are examined the increase in nitrogen is frequently no

more than about 0.005 per cent. Likewise the practice of many workers in this field to report results as milligrams of nitrogen fixed per gram of carbohydrate used is objectionable since it denies the critical reader the original data which constitute the significant observations. It appears, then, that until more impressive evidence is furnished by several independent stations, the role of nonbiological nitrogen fixation in the soil economy should be accepted with considerable reservation.

From Egyptian cotton bolls, rice hulls, and tropical soils, Stapp (25) has isolated a nitrogen fixing organism which he claims represents a new genus, *Azotomonas*. *Azotomonas insolita* is apparently smaller than the described species of the azotobacter and has a lower pH optimum than the azotobacter species other than *A. indicum*. He secured as much as 11.9 mg. of nitrogen fixed per 100 ml. of originally nitrogen-free medium. Plotho (26) has reported the fixation of a substantial amount of nitrogen, 5 mg. per 100 ml. in 28 days, by an actinomycete. He has also isolated an actinomycete from alder and reproduced the alder root tubercles by its addition to plants that had been germinated aseptically (27).

In recent years Schanderl (28, 29, 30) has surveyed the capacity of a variety of biological agents to fix nitrogen, and, contrary to the experience of others, has had little difficulty in demonstrating fixation by several film-forming yeasts, nonleguminous plants, and molds. Not only does Schanderl conclude that nitrogen fixation is rather general among plants and not peculiar to legumes, but he also contends that the chief seat of fixation is in the leaves and not the nodules of leguminous plants. The latter conclusion is at variance with results long accepted by workers in this field, which have shown fixation by legumes to occur predominantly in the roots—lately confirmed by using isotopes (13). Frei (31) also reported nitrogen fixation by several film-forming yeasts and one mold. Bose (32) questioned the reports that many mycorrhizal fungi can fix nitrogen. Of fungi examined he has found fixation only by a few species. In the report of the Imperial Agricultural Chemist (33), Nath repeats his earlier statement that he has secured fixation of nitrogen by maize. Oknina (34) found that oats and barley contained more nitrogen if inoculated with root-nodule bacteria from legumes. Aphids are alleged to fix nitrogen when crushed with oxalacetic acid (35). These reports are in need of verification. Critical studies made with an unequivocal technique such as the isotope tracer method may eliminate many of the claims.

## BIOCHEMISTRY OF THE ORGANISM

*Rhizobium*.—Nielsen and co-workers have published a series of papers on the physiology of the root nodule bacteria. *Rhizobium leguminosarum* assimilated thirty-two of forty amino acids tested; in comparison only twenty-two were assimilated by yeast (36). Single amino acids in several cases supported vigorous growth of the root nodule bacteria. Glycine proved to be inhibitory to the alfalfa organism though satisfactory for other species (37). The alfalfa strain tested required less nitrogen for growth and had a lower nitrogen content than the other organisms. Analysis of cells from a medium containing excess asparagine nitrogen showed 8 to 9 per cent nitrogen with a drop to 6 per cent on aging (38). Cells grown on a medium deficient in nitrogen contained only 3.5 per cent nitrogen.

From a study of the effect of temperature on nine strains of rhizobia, Allison & Minor (39) concluded that optimum growth rates for most of the organisms fell between 29° and 31° C., although *R. meliloti* grew best at 35° C. A somewhat lower temperature, 28° C., was recommended for incubation, as the optimum temperature is rather close to the maximum temperature. The variation in temperature optimum for growth is more marked than the variation in temperature optimum for respiration; six species of the rhizobia showed maximum oxygen uptake on glucose at  $37.5^{\circ} \pm 1^{\circ}$  C. (40). Tam & Wilson (41) found the optimum temperature for methylene blue reduction by good and poor strains of *R. trifolii* and *R. leguminosarum* ranged from 41° to 46° C.

The relative respiration rates of ten strains of rhizobia (40) on a variety of substrates indicated that certain organic acids supported a much more rapid respiration of the "slow-growing" rhizobia (soybean, cowpea organisms) than did carbohydrates, whereas the "fast-growers" oxidized carbohydrates at a rate nearly equivalent to the organic acids. Nielsen & Johansen (42) observed a marked stimulation of growth of the soybean and lupine organisms on addition of citric acid; when glucose was also present citrate was used in preference to it. Other organic acids proved excellent carbon sources for the organisms. Using a photometric method, Tam & Wilson (41) determined the effect of pH, substrates, and inhibitors on the dehydrogenase activities of the rhizobia. Several substrates, including pentoses, polyhydric alcohols, organic acids, and ethyl alcohol were distinctly superior to glucose.

Georgi & Ettinger (43) followed the growth and fermentation of several species of rhizobia and a culture of *Azotobacter chroococcum* on a number of sugars and sugar acids and found the mono- and disaccharides more readily used than the tri- and polysaccharides and the sugar acids. No differences were evident between efficient and inefficient strains of the root nodule bacteria. The rhizobia produce adaptive enzymes for the oxidation of a number of polyhydric alcohols, acetate, and succinate, but their enzymes for the oxidation of carbohydrate are apparently constitutive (44).

Allison and co-workers (45) studied the respiration of intact root nodules and observed that oxygen supply was limited in large nodules as evidenced by a high respiratory quotient. Further investigations (46) indicated that in air the rates of respiration per unit dry weight of legume nodules and small legume and nonlegume roots were nearly the same— $Q_{O_2}$  values of about 2.2. In oxygen the nodules respired approximately twice as rapidly as the roots. They concluded that "the nodule consists of plant cells largely filled with comparatively inactive bacteria." Wilson (2), however, cited data indicating that when the root nodule organisms of soybean and cowpea are freed of nodular debris, their respiration rate is comparable to that of young active organisms from laboratory cultures. Bond (47) also measured the respiration and carbohydrate utilization of intact leguminous plants and found, contrary to Allison and co-workers (46), that respiration per unit dry weight of tissue of the nodules was approximately three times that of the roots. Bond further calculated that during a period from shortly before flowering to the stage of early fruit formation the consumption of carbohydrate within the nodules of the plant was 16 per cent of the total carbohydrate synthesized during the period; 19 mg. of carbohydrate was consumed in the nodules for each milligram of nitrogen fixed. From his estimate that the bacteria accounted for 75 per cent of the respiration of the nodule as a whole, one would conclude that 12 per cent of the carbohydrate synthesized was used to support the nodule bacteria. Bond is in disagreement with Demolon & Dunez (48), who found no effect of the symbiotic organisms on total carbon dioxide output of the root tissues, and with Allison and co-workers (46), who stated that their earlier figure of 3 to 6 per cent for this function should be revised downward to some unstated level. Their values were calculated from the activity of excised nodules, whereas Bond's measurements were on the intact plant. If the nodule on the plant receives dissolved oxygen via its vascular system, its rate

of respiration would be more rapid than that observed with excised nodules, for oxygen diffusion limits nodular respiration.

Thorne & Burris (49) demonstrated that the respiration of root nodule bacteria taken directly from the nodule and bacteria from pure cultures responded to physical and chemical changes in a similar manner. The observation that resting cell suspensions of many bacteria do not oxidize substrates completely but assimilate a portion of the substrate as a polysaccharide has attracted considerable attention. Washed suspensions of *R. trifolii* 205 oxidize only about a fourth of the glucose supplied; the percentage oxidation increases when 2,4-dinitrophenol is added (50). The effect of dinitrophenol can be induced either by adding it initially or after assimilation of glucose is complete. In this instance, therefore, it is unnecessary to postulate an inhibition of assimilation to explain the increased oxidation, for a stimulation of the oxidation of already assimilated material can better explain the observed results.

Subsequent to the demonstration that the chief growth factor for the root nodule bacteria is biotin, Chen & Hsu (51) reported a thermostable factor stimulating the growth of rhizobia. Nielsen & Johansen (52) also ascribed a growth stimulation to a substance other than biotin. Wilson & Wilson (53) tested strains of the alfalfa and clover organisms for their ability to grow in the absence of biotin. Special techniques were adopted to meet the obvious criticism that any observed growth depended on traces of biotin in inoculum or medium. They grew the organisms successfully through numerous transfers and concluded that although most strains of rhizobia can grow through continuous transfer on a medium devoid of biotin, the cell concentration reached is only about a tenth that obtained in the presence of biotin; that some strains make optimal growth in the absence of biotin; and that some entirely fail to grow under such conditions. Synthesis of "coenzyme-R" (biotin) has been demonstrated for a few organisms by Allison & Minor (54), but in general little of the factor is synthesized by the root nodule bacteria.

Lilly & Leonian (55) report that desthiobiotin did not replace biotin in the nutrition of *Lactobacillus casei*, *L. arabinosus*, or *R. trifolii* 205, though it did replace biotin in the nutrition of yeast. It exerted an antibiotin action on *L. casei* but not on *L. arabinosus* and *R. trifolii* 205. These data suggest that the latter two organisms in conjunction with yeast should be of use in distinguishing desthiobiotin from biotin, for they are neither stimulated nor inhibited by desthio-

biotin, whereas yeast and *L. casei* respectively are subject to these effects.

The red pigment of legume root nodules has been described as an oxidation product of dihydroxyphenylalanine and as a hemoprotein. Burris & Haas (56) determined the absorption spectrum of the oxidized and reduced red pigment from cowpea nodules. The method of preparation and the properties of the pigment indicate that it is a protein, and its absorption spectrum is far different from that of the red oxidation product of dihydroxyphenylalanine. Addition of oxidizing and reducing agents reversibly alter the spectrum—a result of oxidation and reduction and not of oxygenation and deoxygenation. In reconstructed diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) systems no reduction of the red pigment was demonstrated, and hence no evidence for a function of the pigment in oxidations via the DPN or TPN systems could be established.

Stimulation in nitrogen fixation by the azotobacter through addition of molybdenum has created interest in its effect on symbiotic nitrogen fixation. Analyses (57, 58) indicated that the concentration of molybdenum was higher in root nodules of legumes than in the roots which in turn was higher than in the aerial parts of the plant. Legume seeds are also relatively high in molybdenum (57). Bertrand (59) states that 0.4 mg. per l. of nutrient solution is the optimum concentration of molybdenum for legumes. Jensen (60) observed no effect of molybdenum on nitrogen fixation by alfalfa or clover. He reported (61) later, however, that more molybdenum was absorbed by plants during fixation of free nitrogen than in utilization of combined nitrogen, and that at concentrations of less than 0.03 to 0.05  $\mu\text{g}$ . molybdenum per plant nitrogen fixation was stimulated on addition of this element. Leroux (62) has observed stimulation of nitrogen fixation by peas upon addition of each of the following: boron, fluorine, arsenic, iodine, chromium, manganese, copper, zinc, and lead. Javillier (63) and Hennig & Villforth (64) have likewise found trace elements helpful in increasing nitrogen fixation in soils and plants.

*Azotobacter*.—Horner & Allison (65) have reviewed the literature on the utilization of nitrogen compounds by the azotobacter and also have reported new studies of their own. Among thirty-five organic compounds tested, only urea, aspartic acid, asparagine, adenine, and glutamic acid were definitely assimilated; the inorganic sources, nitrate, nitrite, and ammonia were readily used. It was questionable whether guanine, allantoin, cytosine, or uramil were utilized, for the

growth was only slight even after extended incubation. The conclusion was drawn that the occurrence and availability of fixed nitrogen compounds in soils are not likely to retard markedly nitrogen fixation.

Wilson and co-workers (66) have used  $N^{15}$  to determine the effect of combined nitrogen on nitrogen fixation by *A. vinelandii*. When combined nitrogen compounds containing normal nitrogen and molecular nitrogen enriched with  $N^{15}$  were supplied simultaneously, the growing cultures could use either or both. Analysis of the cells for  $N^{15}$  indicated exactly how much nitrogen assimilation could be traced to the use of combined nitrogen compounds and how much to the fixation of molecular nitrogen. Only ammonia and urea (which is essentially equivalent to ammonia in the presence of the urease of the cells) were used to the exclusion of molecular nitrogen. Adaptation by previous culture on a medium containing nitrate was necessary before this form of combined nitrogen completely suppressed fixation. No single amino acid nor the mixture from a casein hydrolysate competed effectively with molecular nitrogen in the nutrition of the azotobacter; the amide, asparagine, was used more readily than any of the amino acids, supposedly because of the release of ammonia. Vukhrer (67) has found that on prolonged incubation (ten to twenty-five days), the azotobacter will deaminate glycine as evidenced by the appearance of ammonia and the disappearance of amino nitrogen; in such a period extensive autolysis of the culture would doubtlessly occur.

Washed cells of *A. vinelandii* show adaptation to substrates, for they oxidize sucrose about five times as rapidly when grown on sucrose as when grown on mannitol, and oxidize mannitol about forty times as rapidly when grown on mannitol as when grown on sucrose (44). Fructose is used much more readily than glucose. Acetate, hexose-diphosphate, and pyruvate are excellent substrates for *A. vinelandii*. Xylose and arabinose were oxidized very slowly, which is in agreement with Jensen's (68) observations that xylose and xylan were not used to any extent for growth by the azotobacter. However, Jensen's observation that arabinose is well utilized by *A. vinelandii* indicates a possible difference in the strains of this species employed in the two studies. Fife (69, 70) followed respiration of the azotobacter in a calorimeter, and his measurements indicated complete oxidation of glucose by aerated cultures. In these tests increasing the oxygen tension did not decrease the rate of respiration as reported by Burk (71) for microrespiration studies.

Succinic, lactic, and malic dehydrogenases, hydrogenase, oxal-



acetic acid decarboxylase, and  $\alpha$ -ketoglutaric acid decarboxylase have been obtained in cell-free preparations from the azotobacter (72). No success was had in efforts to obtain nitrogen fixation by such cell-free preparations of these organisms (12).

In order to secure large quantities of the azotobacter cells for cell-free preparations, Lee & Burris (73) studied the growth of the organism in a 200-gallon copper tank used in pilot plant production of yeast. Yields of 10 to 11 lbs. of wet cells could be obtained from 300 l. of nitrogen-free medium. The efficiency of conversion of sugar to cells was about 15 per cent. The cells from a nitrogen-free sucrose medium had a vitamin content equal or superior to that found in yeast; a molasses medium gave higher concentrations of biotin, thiamine, and pantothenic acid than the sucrose medium, but the cells were somewhat lower in nicotinic acid and riboflavin. Starkey (74) found that 71 per cent of the riboflavin produced by *A. vinelandii* was excreted into the medium in a period of ten days. Jones & Greaves (75) tested the effect of a number of vitamins and growth factors on *A. chroococcum* and concluded that although slight benefit occurred at times upon addition of thiamin, riboflavin, or ascorbic acid, there was no evidence that any of the growth factors were essential or materially increased the azotobacter metabolism. Nilsson and co-workers (76, 77) have also been interested in production of azotobacter as a feedstuff because of its high vitamin content and simplicity of the medium used for its growth. Despite its favorable properties, they concluded that it could scarcely compete with *Torula utilis* in view of the low yields and the difficulty experienced in reproducing results.

Allen (78) studied fixation of nitrogen under the conditions employed for aeration in the activated sludge sewage disposal system. By neutralization, addition of salts and carbohydrate, inoculation with activated sludge, and vigorous aeration, active fixation of nitrogen could be induced. The process was continuous and under best conditions gave 7 mg. of nitrogen per gm. of glucose used.

Horner and co-workers (79), in further studies on the influence of molybdenum and vanadium on the azotobacter, have found eight strains of *A. chroococcum* and one strain of *A. vinelandii* that show a tenfold to thirtyfold increase in nitrogen fixation upon addition of molybdenum. Two strains of *A. agile* and three of *A. vinelandii*, which fixed nitrogen quite vigorously in the absence of molybdenum, showed only a twofold increase on its addition. Efficiency of fixation based on carbohydrate consumed by *A. chroococcum* was improved markedly

by addition of molybdenum. An effect on *A. chroococcum* by addition of molybdenum or vanadium could be detected at 0.00001 p.p.m. to 0.0001 p.p.m.; 1 p.p.m. gave maximum growth in six days. Vanadium gave only 50 to 80 per cent the stimulation produced by molybdenum, and stimulation from addition of tungsten salts was attributed to molybdenum impurities. Although it has been reported that manganese can replace magnesium in the growth of the azotobacter (80), salts of this element had no appreciable effect on the azotobacter in the presence of the usual magnesium concentration of the medium (79). Lind & Wilson (81) found that the stimulation in nitrogen fixation of *A. vinelandii* by the presence of a contaminating aerobic spore-former could be attributed to mobilization of unavailable iron by the contaminant. Such a relationship between organisms might well exist under natural soil conditions. Lewis (82) observed that copper lengthened the lag growth phase in azotobacter cultures and that this effect was reduced by iodine.

#### MECHANISM OF FIXATION

*Chemical mechanism.*—The chemical mechanism of biological nitrogen fixation has been long the subject of frequent speculation, but only within the past decade has experimental evidence in favor of various schemes been furnished. Figure 1 summarizes the major stepwise oxidations, reductions and hydrations of the nitrogen molecule which might lead to the final product of fixation—the amino acid in the protein molecule. Obviously, many of the intermediates suggested in this scheme would exist only momentarily, but others should be detectable.

The oxidative pathways of nitrogen fixation have had few supporters in recent years, though they are attractive from the viewpoint of thermodynamics because the reactions involved are exothermic. The inert nature of nitrogen and the difficulty of splitting its stable molecular linkage are considerations which favor an initial step that would be energetically easy to perform. This initial easy pathway, however, constitutes no over-all energy bargain, for the nitrogen in amino acids is fully reduced and eventually the compounds must be reduced to this status. Experimental evidence to support an oxidative pathway of nitrogen fixation is meager at best, and consists chiefly of reports of the presence of small amounts of nitrate or nitrite ions in the azotobacter cultures fixing molecular nitrogen. Blom (83) ob-

jects to a primary oxidative step in nitrogen fixation as quite unlikely for a strictly anaerobic organism such as *Clostridium pasteurianum*. Since as yet no experimental evidence exists to indicate that anaerobic and aerobic fixation follow the same route, Blom's objection is not necessarily apropos to aerobic fixation.

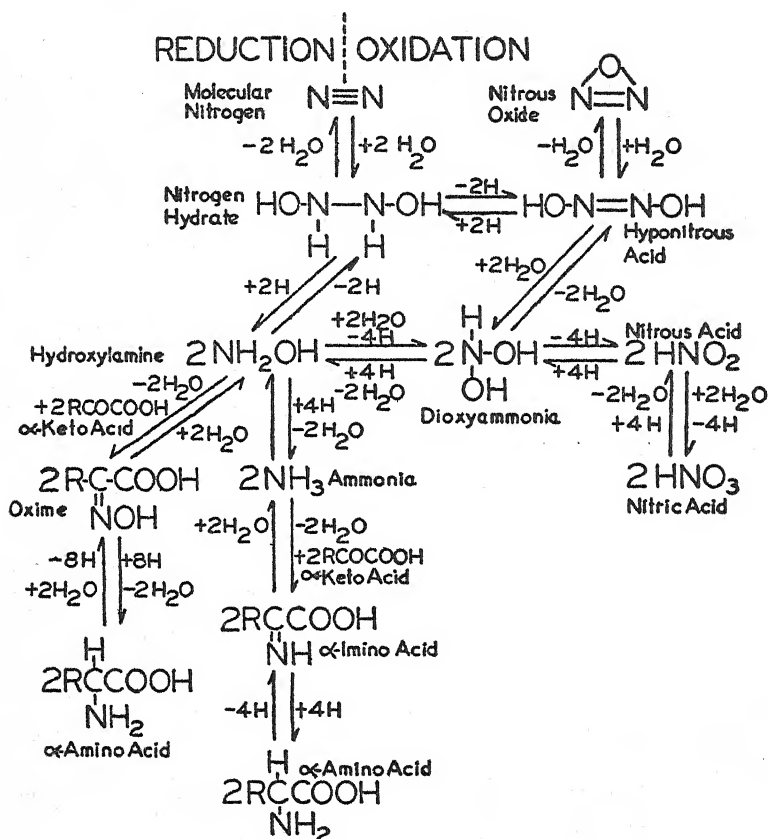


FIG. 1.—Theoretical pathways of nitrogen fixation

The proponents of reductive nitrogen fixation represent two groups, those favoring hydroxylamine and those favoring ammonia as the key compound in fixation. Virtanen (5, 84) has proposed that nitrogen is fixed as hydroxylamine, through some unknown intermediate compound, and combines with oxalacetic acid to form oximi-

nosuccinic acid, which in turn is reduced to aspartic acid. He has unquestionably presented the most extensive supporting evidence to date, but as already discussed by Wilson (2) most of the evidence is not specific, i.e., does not exclude the other hypotheses.

The evidence cited by Virtanen in support of the hydroxylamine hypothesis is largely based on studies of nitrogen excretion by leguminous plants. To date, most investigators (85 to 89) in other stations have been unable to obtain excretion of the order of that observed at Helsinki, and hence it has been impossible for others to isolate excretion products to confirm Virtanen's findings. Damodaran & Varma (90), however, have recently reported the extraction of 4 to 21 mg. of hydroxylamine from 100 gm. of acid hydrolyzed legume nodules; a colorimetric method was employed. It would be interesting to have data on the hydroxylamine content of other leguminous and nonleguminous plant parts subjected to the same drastic treatment.

A major support offered by Virtanen for the hydroxylamine hypothesis has been the relatively high level of oxalacetic acid he has found in leguminous plants. Subsequent to our earlier reviews (1, 2), Virtanen and co-workers (91, 92) have published further data on the oxalacetic acid content of leguminous plants. They earlier stated (84), "In the leaves of pea plants taken from different experiments we found 0.5-1 mg. oxalacetic acid per 1 g. of fresh material, corresponding to about 0.1 per cent. oxalacetic acid in the plant sap." In their most recent report (92) it is pointed out that the values given in the above quotation are tenfold too high as a result of misplaced decimal points, and new values cited are in the range from 24 to 94  $\mu$ g. of oxalacetic acid per gm. fresh weight of legume tissue. The content of oxalacetic acid was found to vary with illumination and disappeared after the plant was held for one to two days in the dark. It is of interest that oats contained 13  $\mu$ g. of oxalacetic acid per gm. fresh tissue, and that clover and alfalfa had levels of  $\alpha$ -ketoglutaric acid comparable to the reported concentration of oxalacetic acid.<sup>1</sup>

<sup>1</sup> The report of Wyss, Burris & Wilson (95) that they were unable to find any oxalacetic acid in plant tissue has been criticized by Virtanen (5) on the ground that oxalacetic acid would be destroyed under the acid conditions employed. This criticism first neglects to consider that added oxalacetic acid could be virtually recovered quantitatively; and second, that oxalacetic acid is stable under the conditions used, viz., pH <1, temperature <5° C. Our preference for acid conditions is based upon convenience and the fact that addition of strong alkali (it should be carbonate-free) at the time of grinding of plant tissue is

Virtanen's final line of evidence in support of the hydroxylamine hypothesis, that excised nodules fix nitrogen when oxalacetic acid is added, has not been satisfactorily confirmed in other laboratories. Allison, Hoover & Minor (4) were unable to demonstrate fixation by excised nodules; the small and irregular fixation detected with isotopes (12) was not uniformly correlated with the presence or absence of oxalacetic acid.

The ammonia hypothesis of nitrogen fixation has received further emphasis from Winogradsky (93, 94), but the new evidence can scarcely be considered as critical. The ammonia that appears in the azotobacter cultures (93) is said to be synthesized by a special enzyme, azohydase, and not to arise from deamination. It is postulated that active hydrogen derived from dehydrogenation reactions is used to reduce molecular nitrogen to ammonia, and that the reaction can continue for an extended period after the death of the cells. Dried nodules (94) placed in a closed container near a dish of sulfuric acid were found to give off a quantity of ammonia exceeding the change in total nitrogen of the nodules. Sampling errors unquestionably impose a great limitation on such experiments. That excised nodules fix appreciable amounts of nitrogen under the best conditions is contrary to most experience; that they should fix nitrogen over an extended period in a dried condition is distinctly more difficult to concede. There has been a further report (96) that *A. chroococcum* produces ammonia as one of the first products of fixation.

Recent observations on the nitrogen metabolism of *A. vinelandii* made with the aid of  $N^{15}$  are compatible with and appear to favor the ammonia hypothesis of nitrogen fixation; it is not claimed that they establish the ammonia hypothesis or define it as an exclusive pathway of fixation. A culture of *A. vinelandii* (97), which had grown in normal nitrogen for eighteen hours, was furnished for ninety minutes

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inevitably accompanied by absorption of carbon dioxide; unless care is taken to discharge this carbonate from the inhomogeneous mass of plant tissue, it may be released during the period of measurement and give spurious results. We have made numerous analyses of a variety of plants without finding oxalacetic acid present, this despite the fact that oxalacetic acid added at any stage could be recovered quantitatively at a level of 25  $\mu$ g and detected at 10  $\mu$ g. The reason for the discrepancy with Virtanen's results is not at once apparent, and we must for the moment attribute it to difference in environmental conditions under which the plants were grown. It should not be overlooked, moreover, that occurrence of oxalacetic acid is not specific evidence for hydroxylamine as an intermediate.

with molecular nitrogen enriched with  $N^{15}$ ; at the end of this period the cells were immediately harvested, hydrolyzed, and then fractionated. The highest level of  $N^{15}$  in the amino acids appeared in glutamic acid and the next highest in the aspartic acid fraction. This confirms the important roles of the dicarboxylic amino acids in the nitrogen metabolism of the azotobacter and singles out glutamic acid as the key compound in the fixation mechanism. Long before these experiments were performed Virtanen (5) observed,

Aspartic and glutamic acids are the fundamental amino acids from which the amino group is transformed to other keto acids. Glutamic acid is formed from ammonia and ketoglutaric acid through the effect of glutamic acid dehydrogenase . . . .

That glutamic acid is not formed [in the excretion products] seems to be ascribable to the fact that the reaction velocity of hydroxylamine with oxalacetic acid is many times greater than with ketoglutaric acid. Should nitrogen fixation occur through the ammonia stage, the formation of glutamic acid together with aspartic acid would be very likely.

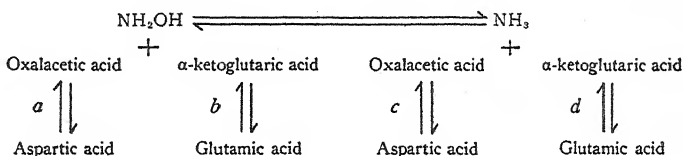
Unless the quite illogical position is assumed that glutamic acid suddenly accumulated more rapidly during the ninety minute period of exposure to  $N^{15}$  than during earlier growth under normal nitrogen, the concentration of  $N^{15}$  in glutamic acid must signify the close proximity of this amino acid in its metabolism to the primary source of  $N^{15}$ , i.e., the molecular  $N^{15}$  supplied. Thus the studies with isotopes favor the primary formation of glutamic acid—and by implication—ammonia.

Additional supporting evidence for the role of ammonia is furnished by other experiments with  $N^{15}$ . If the same type of experiment is performed [unpublished data and abstract (98)] supplying ammonia enriched with  $N^{15}$  rather than enriched molecular nitrogen, the distribution of  $N^{15}$  among the amino acids is almost identical with that noted when molecular  $N^{15}$  was fixed. If a culture of *A. vinelandii* is grown for eighteen hours on normal molecular nitrogen and an ammonium salt enriched with  $N^{15}$  is then added to give a concentration of 10 p.p.m. ammonium nitrogen,  $N^{15}$  can be found in the cells within one minute after its addition as ammonia (98). Further, the rate at which the  $N^{15}$  is taken up shows that it is used to the exclusion of molecular nitrogen. Thus, when ammonia is added to a culture of the azotobacter fixing molecular nitrogen the conversion to ammonia metabolism is not only immediate but is complete. When labeled nitrate is added under the same conditions,  $N^{15}$  cannot be detected in the cells for thirty

minutes, and from that time its uptake proceeds at an increasing rate, whereas ammonia uptake is at a linear rate from the time of its addition. Introduction of ammonia into a system fixing molecular nitrogen constitutes no disruption in the incorporation of inorganic nitrogen into amino acids, for ammonia immediately takes the place of molecular nitrogen; utilization of nitrate nitrogen is subject to a period of adaptation, presumably the formation of enzymes to reduce nitrate to a form suitable for amino acid synthesis. Experiments of the nature described have not as yet been performed with hydroxylamine enriched with  $N^{15}$ , because the toxicity of hydroxylamine precludes its use in comparable experiments. However, continuous addition of low concentrations of labeled hydroxylamine should prove feasible, and observation of its action is of obvious importance.

Is it possible to reconcile the results from the two types of study? It should be noted that the experiments with isotopes were made primarily with *A. vinelandii* and the excretion experiments with leguminous plants. This choice of agent, however, we do not believe to be responsible since other studies have pointed to a close similarity in the fixation mechanism. Study of Figure 1 suggests that the two compounds may be part of the same mechanism in which the precise pathway eventually is dictated by the availability of the carbon chain functioning as the acceptor of fixed nitrogen. Hydroxylamine is separated from ammonia by a reductive step. Since it is most probable that reduction of nitrogen is a stepwise process, hydroxylamine would logically occur in the production of ammonia. From an energetics standpoint the pathways to an amino acid are equivalent, for if hydroxylamine is converted to an oxime it requires a reductive step in addition to that required for formation of an amino acid via ammonia and a keto acid. Amino acid formation either from hydroxylamine via the oxime or from ammonia via the  $\alpha$ -imino acid appears feasible, and there is no reason to assume that the pathways are mutually exclusive. Proponents of the various schemes of nitrogen fixation are interested in the first product of fixation but are more particularly interested in the key nitrogen compound which combines with a carbon chain. Under particular conditions conducive to the formation of large quantities of oxalacetic acid the fixed nitrogen may be predominantly arrested at the hydroxylamine stage with the formation of oximinisuccinic acid which on reduction yields aspartic acid. On the other hand, in the absence of appreciable quantities of oxalacetic acid the reduction may proceed largely to ammonia which after combination with  $\alpha$ -ketoglutaric acid

and reduction gives glutamic acid. These reactions may be considered to be in equilibrium:



Reactions *a* and *d* are likely dominant. Virtanen's data, demonstrating excretion of aspartic acid, isolation of oximinosuccinic acid, occurrence of oxalacetic acid, and fixation by excised nodules, have been used exclusively to favor the functioning of reaction *a* although strictly speaking only the isolation of oximinosuccinic acid is specific for the scheme. The studies with isotopes to date are strictly compatible with the reaction *d* and highly suggestive of its dominance, but they are not critical in the sense that they establish the formation of ammonia to the exclusion of hydroxylamine. Likewise, the fact that workers at other stations have been unable to duplicate Virtanen's results with respect to excretion, occurrence of oxalacetic acid, and fixation by excised nodules plus oxalacetic acid may point only to a fundamental difference in the plants grown in Finland and at these stations. Such differences may quite conceivably shift the balance in the plant metabolism to favor fixation via oxalacetic acid and hydroxylamine. At the same time fixation via ammonia and  $\alpha$ -ketoglutaric acid is not excluded and probably predominates in most agricultural regions where excretion has been shown to be the exception rather than the rule.

*Enzymatic mechanism.*—Investigation of the mechanism of biological nitrogen fixation includes more than consideration of what compounds occur as intermediates in the process. Just as in biological oxidations one is not content with a statement that glucose oxidation gives rise to carbon dioxide and water, so with nitrogen fixation the question arises, what is the enzymatic mechanism involved in the formation of fixed nitrogen compounds including the first product of fixation and the intermediates between it and the nitrogen compound which combines with a carbon chain.

The relation between the nitrogen fixing enzyme and its specific substrate,  $\text{N}_2$ , is obviously of fundamental importance. In this relation the dissociation constant of the enzyme-substrate complex in nitrogen



fixation by *A. vinelandii* has been determined by a number of methods (99). Consideration of all estimates led to the conclusion that the half maximum rate of nitrogen fixation occurs at a partial pressure of nitrogen of  $0.02 \pm 0.005$  atm. Under almost any conditions encountered in nature an enzyme with such a Michaelis constant would have sufficient nitrogen to perform at a nearly maximum rate.

Biological fixation of nitrogen is specifically inhibited by hydrogen, i.e., the inhibition by hydrogen occurs when molecular nitrogen is being fixed but not when combined nitrogen compounds are being used. By estimating rates of fixation under conditions such that the inhibitor, molecular hydrogen, is held constant and the substrate, molecular nitrogen, is varied, and vice versa, it is possible to determine whether the inhibitor is acting in a competitive or a noncompetitive fashion. Data obtained with red clover indicate that hydrogen acts as a competitive inhibitor of nitrogen fixation (100). Wyss & Wilson (101) established that hydrogen is also a specific inhibitor of nonsymbiotic fixation by the azotobacter, and later experiments (102) showed the inhibition to be competitive and reversible. Hydrogen also specifically inhibits nitrogen fixation by the alga *Nostoc muscorum* (103).

Carbon monoxide in the very low concentrations of 0.0001 to 0.0005 atm. specifically inhibits nitrogen fixation by red clover (104) and in about tenfold these concentrations inhibits the azotobacter (105, 106). Fixation by *Nostoc* is intermediate in its sensitivity to carbon monoxide (107). The specificity of carbon monoxide inhibition is not as clear-cut as that of hydrogen, for at the levels of carbon monoxide giving nearly complete inhibition of molecular nitrogen fixation there is a slight but reproducible inhibition of nitrate assimilation by red clover, the azotobacter, and *Nostoc muscorum*. Further increases in carbon monoxide concentration produce a general growth inhibition which is observed even when ammonia is furnished. Inhibition by carbon monoxide may be described as specific in a quantitative sense as distinguished from the qualitative specificity of hydrogen.

As earlier work with symbiotic nitrogen fixation (104) had suggested that inhibition by carbon monoxide was noncompetitive, micro-respiration experiments with the azotobacter (which are more readily replicated) were used in the further elucidation of the nature of this inhibition. Ebersole and co-workers (108) have outlined methods for determining the type of inhibition and have applied these to the results of the azotobacter trials. Analysis of the data showed that carbon monoxide inhibition was primarily noncompetitive, but there was some

evidence of an accompanying competitive inhibition. The noncompetitive nature of the inhibition by carbon monoxide is rather surprising, because the closely analogous molecular structure of carbon monoxide and nitrogen tempts one to the *a priori* assumption of a competitive inhibition.

The fact that nitrogen fixation by leguminous plants, the azotobacter, and the alga, *Nostoc muscorum*, is specifically inhibited by both hydrogen and carbon monoxide indicates a considerable unity in the enzymatic mechanism of nitrogen fixation by these diverse forms. Carbon monoxide, of course, is an inhibitor of other enzymatic systems, but its inhibition of nitrogen fixation is noteworthy because of its effect in such low concentrations.

The presence of the enzyme hydrogenase in the azotobacter has been reported (109, 110). Presence of the enzyme in bacteria taken from pea nodules but its absence in the organism grown on laboratory culture media suggested an importance of the enzyme for the nitrogen fixation reaction. Later and more complete work (111) failed to substantiate the presence of hydrogenase in root nodules or bacteria from the nodules. This does not prove, however, that no association exists between symbiotic nitrogen fixation and hydrogenase activity. Since neither excised nodules nor the bacteria from nodules have been found to fix nitrogen to any extent under the conditions which necessarily are employed in these experiments, it is conceivable that their hydrogenase activity and nitrogen fixing capacity disappear simultaneously.

The hydrogenase from the azotobacter has an optimum pH of 7.5 and an optimum temperature of 40° C. (112). Dependence of rate of reaction on either oxygen or hydrogen tension varies with the number of cells per unit volume, but in each case, with usual conditions, saturation of the enzyme occurs below 0.05 atm. Gas uptake in a hydrogen and oxygen gas mixture by intact cells and cell-free preparations of hydrogenase from the azotobacter consists primarily in the oxidation of hydrogen to water (113). Molecular nitrogen does not inhibit the oxidation of hydrogen, but the oxidation is inhibited by cyanide and carbon monoxide, the hydrogenase system being more sensitive to carbon monoxide than the respiratory system (114). Certain inhibitors, e.g., sodium azide, hydroxylamine, sodium iodoacetate and sodium fluoride, are more active on the total respiration of the azotobacter than on hydrogenase. These inhibitors can be used to suppress endogenous oxidation, in material such as nodules, to aid in the detection of hydrogenase activity. The fact that hydroxylamine in-

hibits respiration to a greater extent than oxidation of hydrogen by the azotobacter illuminates some curious findings of Kubo (115). Observing that gas uptake by *A. chroococcum* on mannitol was decreased by hydroxylamine in air but not in a hydrogen-oxygen mixture, he postulated that this compound is a specific inhibitor for nitrogen fixation. It is more likely that Kubo was dealing with the differential effect of hydroxylamine on the hydrogenase and respiratory systems in the azotobacter; hence this possible support of the hydroxylamine hypothesis should be discarded (114).

Sources of combined nitrogen which readily inhibit nitrogen fixation by the azotobacter also inhibit formation of hydrogenase (116). The adaptation of the azotobacter to nitrate nitrogen increases the effectiveness of nitrate in inhibiting both nitrogen fixation and hydrogenase formation. When the organism is supplied with combined nitrogen and cultured in an atmosphere of hydrogen and oxygen, its hydrogenase content is low despite the fact that the specific substrate hydrogen is present. Formation of hydrogenase in the azotobacter appears more responsive to the presence of nitrogen than to hydrogen. Lee & Wilson (116) have reviewed the evidence and concluded that hydrogenase is likely closely associated with the nitrogen fixing system in the azotobacter; they have suggested that the systems responsible for oxidation of hydrogen and fixation of nitrogen may possess common components. That most extensive formation of hydrogenase occurs only when the azotobacter are actively fixing nitrogen suggests a close interdependence of hydrogenase and the nitrogen fixing system and poses an intriguing problem for further study.

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DEPARTMENTS OF BIOCHEMISTRY AND AGRICULTURAL BACTERIOLOGY  
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# MINERAL NUTRITION OF PLANTS

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In common with previous reviewers, the author has made no attempt to present complete coverage of the current year's contributions. The subjects reviewed in the present paper are: absorption and accumulation of ions, list of essential elements, functional aspects in plant nutrition and ion interrelationships, salt tolerance of plants, nutrition in relation to yield and fruit quality, tissue testing for the diagnosis of nutrient deficiencies, and relation of nutrition to disease resistance.

Of the publications which came to the writer's attention in 1944, special mention should be made of a series of lectures in book form by D. R. Hoagland (1) entitled "Lectures on the Inorganic Nutrition of Plants." The aim of this publication, as stated by the author, is "to present a general perspective of several important aspects of the field of plant nutrition, with a broad interpretation of this term in mind." The student in search of orientation in the broad and complex field of plant nutrition and soil-plant interrelations will be greatly aided by the clear and concise presentation, and discussions found in this work.

## ABSORPTION AND ACCUMULATION OF IONS

It is now well-established that the absorption and accumulation of salts by plant roots is linked with aerobic metabolism and as indicated by Hoagland (1) further progress in an understanding of the mechanisms involved is most likely to be found in the biological reactions which take place in plant roots. In this connection, Hoagland (1) reviews and discusses briefly the possible relationships of protein synthesis, organic acid metabolism, phosphorylation, and hormone activity to salt accumulation. In order to secure further evidence along these lines, Machlis (2) studied the effects of certain respiratory inhibitors, namely, potassium cyanide, sodium azide, potassium iodoacetate, and potassium malonate on respiration and on the intake of radioactive bromide by excised barley roots. He found that, at certain concentrations, all of these greatly depressed both bromide accumulation and respiration. He also found that the inhibiting effect of malonate and iodoacetate could be largely overcome by the addition of cer-



tain organic acids. These results support the postulate that an organic acid respiratory cycle is intimately linked with salt accumulation. Cytochrome oxidase is the oxygen activating enzyme probably affected by the cyanide and azide while the iodoacetate and malonate block another part of the organic acid cycle. When sufficient other organic acids are added, the inhibitory effects of the latter compounds are overcome.

In another paper, Machlis (3) showed a gradient in respiratory activity in excised barley roots, the respiratory rate decreasing with increasing distance from the root apex. This fits in with other work showing similar gradients in salt absorption.

As regards certain aspects of the mechanism of salt absorption, Hoagland (1) concludes that "there occurs apparently some preliminary combination of protoplasmic constituents with the solute and it is almost impossible to avoid some concept of ion exchange as part of the process of salt accumulation." Lundegårdh & Stenlid (4), in a short paper, postulate that the protoplasmic membrane behaves as an amphoteric colloid with pronounced acid dissociation. The hypothetical acid ( $R^-$ ) reacts with neutral salts according to the equation  $H^+R^- + M^+A^- = H^+A^- + M^+R^-$  following the law of mass action. The acid constituent of the protoplasmic membrane is supposed to be phosphoric acid in organic linkage and in the paper cited, evidence for the exudation of adenosine phosphoric acid ( $1-3 PO_4$ ) and a flavanone by plant roots is given. The presence of these compounds in the protoplasmic membrane suggests functional activity of several kinds in connection with salt accumulation.

#### LIST OF ESSENTIAL ELEMENTS

The essentiality of boron, manganese, and zinc for higher plants is conceded by most if not all investigators. There is also strong evidence that copper is essential though doubt has been expressed by some. Recently the author (5) has been able to produce typical copper deficiency symptoms in lemon cuttings growing in purified cultures especially prepared to exclude copper. The copper content of the foliage was of the same order of magnitude as that of citrus trees in the field showing copper deficiency (exanthema) symptoms.

Since the last review on this subject, Stephens & Oertel (6) have recorded responses of subterranean clover, perennial rye grass, and white clover to small additions of molybdenum to an Australian soil.

Thus, evidence continues to accumulate that molybdenum is an essential plant-food element (7, 8).

The work of Steinberg (9, 10), suggesting the essentiality of gallium for *Aspergillus niger*, coupled with the finding of traces of this element in certain California soils, stimulated Liebig, Vanselow & Chapman (11) to determine whether evidence for its essentiality in citrus could be uncovered. The results were negative and it was concluded on the basis of spectrochemical analysis of various parts of the plant and of culture solutions that if this element is essential the need of citrus is met by amounts less than one part per billion in the culture solution. The lemon plants grown in control cultures were green, healthy, and vigorous, and no gallium could be detected spectrographically in any part of the plant. As little as 1 p.p.m. gallium (dry weight) will show up in spectrograms of citrus ash, and since none was found, it is evident that if this element is essential for healthy growth in citrus, amounts less than this are ample. Similar conclusions were reached for the element indium.

Tauböck (12) studied the aluminum requirements of many kinds of plants. He used vessels of synthetic material or paraffin coated stoneware, and water three times distilled and condensed in a quartz condenser. Without aluminum there was cessation of growth in a few weeks and progressive necrosis. Evidence of this kind, however, is not necessarily conclusive as regards essentiality. In previous work with this element using lemon and orange cuttings, Liebig, Vanselow & Chapman (13) obtained marked top and root growth responses by the addition of 0.1 p.p.m. aluminum to solution cultures. However, it was found that the poorer growth of the plants without aluminum was due to the presence of a slight excess of copper and that equally good growth could be obtained by reducing the copper content of the solution. Where copper was below toxic levels, no stimulating effect from aluminum was obtained.

In work with the organism *Escherichia coli*, Young, Begg & Pentz (14), using purified culture media, found no evidence of essentiality for the elements strontium, cobalt, nickel, aluminum, lead, caesium, silicon, and molybdenum.

In connection with sodium, in its relation to potassium, Richards (15) has critically reviewed the literature and expressed the opinion that

Sodium cannot perform the primary essential function of potassium. When the potassium level is too low to exert this function adequately, toxic accumulation

of other elements may occur, producing characteristic symptoms and reducing growth still further, or even leading to death of the plant. Sodium may hinder or entirely suppress such accumulations, thus improving the general condition; it may also increase phosphorus uptake, with either favorable or detrimental results. In some plants, e.g., barley, however, sodium may itself accumulate to toxic levels, resulting in secondary injury of a modified type; but in others (beet, mangold) higher internal concentrations are tolerated and considerable improvement results.

#### FUNCTIONAL ASPECTS IN PLANT NUTRITION AND ION INTERRELATIONSHIPS

Progress in this broad and complicated field is understandably slow and while there is much general and suggestive information regarding the functions of most of the known essential elements, the details are largely obscure and are likely to become clear only as knowledge of plant biochemistry develops, and various steps in the chain of processes involved in vital activity, growth, differentiation, reproduction, etc., are linked into a connecting whole.

*Boron.*—No clear evidence of boron function is as yet at hand beyond the many observations of the effect of its lack in meristematic tissues, and interrelations with calcium. Walker (16) has described in some detail the histological changes in beet roots when boron is deficient. In the meristematic region of the beet root, cell division and cell size are increased when boron is lacking but cell differentiation is decreased, resulting in a reduction of xylem and phloem tissue. Necrosis of these large cells follows and the toxic products of this necrosis probably affect adjacent tissue. This breakdown of conductive tissue is responsible for some of the visible manifestations of boron deficiency and no doubt for many secondary effects such as accumulation of organic and inorganic substances in various parts of the plant. This needs to be kept in mind when the attempt is made to connect these accumulations with some essential function of boron in a biochemical process.

While the similarities of boron to calcium deficiency continue to be emphasized by many, the author's work with citrus indicates no great similarity beyond the observation that meristematic regions appear to be the first affected. Foliage and fruit symptoms are quite different for boron and for calcium deficiency, respectively.

The assumption that calcium supply influences boron absorption continues to receive additional support. In sand culture work with

tomatoes, Reeve & Shive (17) showed that increased calcium in the nutrient solution aggravated boron deficiency where boron was low, and decreased boron toxicity where the level of this element was high. Variations in potassium also influenced boron deficiency and toxicity symptoms but these are interpreted as indirect and due to the effects of potassium on calcium absorption.

In pot experiments with soil using alfalfa, oats, and tobacco, Jones & Scarseth (18) got further evidence that calcium influences boron absorption. White-Stevens & Wessels (19), in sand culture experiments with broccoli, found, under certain conditions of nitrogen and phosphorus supply, that increased potassium aggravated boron deficiency, a finding similar to that reported by Reeve & Shive (17) but not explicable on the calcium relation hypothesis. Scripture & McHargue (20) obtained in nutrient culture some evidence that boron increased protein synthesis, a suggestion in line with that offered by others. In nutritional experiments with tomatoes, Beckenbach (21) found that high nitrogen aggravated boron deficiency, which finding he interprets as suggesting an association of boron with protein synthesis. Tomatoes deficient in phosphate required more boron than those receiving ample phosphate. This led to the postulate that boron may function interchangeably with phosphorus in certain reactions.

To determine the extent to which the inorganic composition of tomatoes could be influenced by boron supply, Parks, Lyon & Hood (22) grew this plant in sand cultures supplied with a uniform base nutrient solution in which boron was varied from 0.5 to 60.5 p.p.m. Considerable differences in nitrogen, magnesium, molybdenum, manganese, iron, calcium, phosphorus, zinc, copper, cobalt, sulfur, and potassium were found in the tomato leaflets as the boron supply was increased. In discussing the variability of results reported by different investigators, the point is made that either decreases or increases of a given element may result from boron variations, depending upon the basis of comparison and the particular range of boron concentrations compared.

Bailey & McHargue (23) studied the catalase, peroxidase, oxidase, and invertase activities of tissues from tomato plants grown in nutrient solutions supplied with concentrations of boron ranging from 0.1 to 1 p.p.m. In general, enzyme activity increased with increase in boron concentration but this is interpreted as reflecting a generally improved metabolic condition of the plant rather than a direct influence of the boron on the enzymes.

Moinat (24) conducted nutrition experiments with lettuce to determine whether spraying indole acetic acid on the foliage could take the place of boron. While this treatment affected the growth, character, and color of the lettuce, no evidence of a replacing effect for boron was obtained.

In a study extending over four years and including fifty-eight species of plants, Eaton (25) made some interesting observations on the nutritional effects of boron in concentrations ranging from 0.03 to 25 p.p.m. when supplied to outdoor sand cultures. In most of the plants (stone fruits an exception), boron accumulates in the leaves in soluble but largely immobile form. He suggests that the boron becomes attached to some large molecule which, though soluble, is unable to pass through the plasma membranes of mesophyll cells. Owing to the immobility of boron in leaf tissue, plants may show symptoms of boron excess in old leaves and yet not be supplied with excess, perhaps not enough, boron in meristematic regions. Thus, there is some overlapping of beneficial and toxic effects in the same plant. Noting some signs of seasonal variation in this behavior, he postulates that high light intensity may be responsible in part for boron immobility in leaves. As a result of the boron-accumulating property of leaves, leaf analysis of such plants may not be a particularly accurate indication of boron adequacy or inadequacy.

In none of the current literature on boron can one glean much as to the real function of this element in the plant. Protein and carbohydrate metabolism occupy such central positions in plant activity that one can scarcely conceive of any of the mineral nutrients not being connected directly or indirectly in one or more of the reactions involved in the synthesis or transformation of these.

*Iron.*—Evidence that iron is a part of various enzyme molecules continues to accumulate. Waring & Werkman (26) grew *Aerobacter indologenes* in iron deficient nutrient media and showed that catalase, peroxidase, formic dehydrogenase, and hydrogenase activities were suppressed by iron deficiency.

Lewis (27) found with the yeast *Torulopsis utilis* that lack of iron resulted in increased synthesis of thiamine, riboflavin, nicotinic acid, pyridoxine, and pyridoxine isotels,<sup>1</sup> but decreased the rate of synthesis of biotin, inositol, *p*-aminobenzoic acid, and Norit-eluate factor isotels. While this work was carried out from the point of view of determining

<sup>1</sup> The term "isotels" designates compounds able to perform the same function.

the effects of trace elements on vitamin synthesis, it is suggestive in connection with iron function.

Glenister (28) measured respiration rates of iron deficient and green sunflower leaves from plants grown in nutrient media. Respiration rate was less in the iron deficient leaves but the green leaves from lower down on the stem of the iron deficient plants produced carbon dioxide at the same rate as comparable leaves from plants not deficient in iron. Though the iron in these old leaves is immobile, it apparently continues to function in both chlorophyll formation and the respiratory enzymes of which iron is a component. The iron content of the terminal chlorotic leaves was less than in the green plant and the author suggests that such iron as is present is rendered inactive because manganese is too high in relation to the iron. The excess manganese prevents the normal cyclic valence changes in the iron containing enzymes. Though no manganese determinations were made, this component was added to the nutrient solutions and it is reasonable to suppose that the manganese-iron ratio would be higher in the chlorotic leaves than in the green leaves. The findings of Somers, Gilbert & Shive (29) and Somers & Shive (30), that it is the balance between manganese and iron which determines the functional activity of each, lends support to Glenister's reasoning.

In studies of lime-induced chlorosis, Lindner & Harley (31) analyzed green and chlorotic leaves of pear, apple, apricot, peach, and cherry trees growing on high lime soils and found in conformity with results reported by others that the iron content in many cases was no lower in chlorotic than in green leaves and that the potassium of chlorotic leaves was high and the calcium low. They postulate that perhaps the excess potassium absorbed in high lime soils may be the cause and not the result of iron chlorosis. The suggestion is made that potassium may displace iron from the enzymes involved in chlorophyll formation. Further work to determine the cause or causes of lime-induced chlorosis has been reported by Thorne & Wallace (32). These workers place emphasis on the factors both in the plant and in the soil which affect the balance between ferrous and ferric iron, iron deficiency being associated with the ferric form. These investigators found, in general, more total iron and more iron soluble in 1 *N* hydrochloric acid in green than in chlorotic leaves; also, more ferrous iron was found in green than in chlorotic leaves. In soils from chlorotic and nonchlorotic areas more easily reducible iron was found in the latter than in the former. These data support the view that both soil and conditions

within the plant are involved in iron chlorosis. In this connection, the writer has repeatedly observed in culture solutions containing ample soluble iron, that iron chlorosis of citrus can be brought on by a variety of conditions. With citrus trees growing out of doors in solution cultures maintained at approximately pH 4.0 and supplied with ample iron, potassium deficiency brings on typical iron chlorosis in the leaves. Prolonged magnesium deficiency under similar conditions also produces iron chlorotic leaves. Lemon trees supplied with a nutrient solution low in calcium and high in potassium also became iron deficient. Slight excesses of copper and zinc in the nutrient solution will cause iron chlorosis in citrus. The author has also observed in outdoor citrus cultures that more iron chlorosis appears in winter than in summer. This is a general observation in the field also. The author has also confirmed, with citrus, Olsen's (33) finding that under neutral or slightly alkaline culture conditions high phosphate will bring on iron chlorosis. The foregoing observations indicate that nutritional disturbances of many kinds influence iron availability. This leads to the suggestion that not one but many factors may upset the normal iron metabolism in the plant. However, the balance between ferric and ferrous iron may well be very important and it is apparent that a considerable number of nutritional disturbances may upset this balance.

Some of the same factors which operate in the plant to make iron unavailable are probably operative in the soil. In recent work, Guest (34) has confirmed and added to some earlier observations by the author (35) that the number of local zone contacts between absorbing roots and iron-bearing minerals is a factor. In paired sand cultures containing the same quantity of calcium carbonate and magnetite and supplied with a periodically circulating nutrient solution from a common reservoir, it was found that where the magnetite had been ground in a ball mill no iron chlorosis developed, whereas the original unground form not as finely divided was inadequate. This work clearly indicates that the amount and state of division of iron-bearing minerals in the soil may be another external factor in iron deficiency.

*Zinc.*—In a further study of the relation of zinc to seed production, Reed (36) grew pea plants in nutrient media of graded zinc contents and made a microscopic study of the ovaries and anthers of the healthy and zinc deficient plants. Pollen formation was not affected by zinc deficiency nor was pollen viability. However, the ovules, instead of developing normally, were misshapen and failed to grow in the normal manner, suggestive of a lack of some specific organizing substance.

These observations are submitted as further evidence of the intimate relation of zinc to fundamental processes involved in cellular metabolism and respiration.

*Sulfur*.—Continuing their work on sulfur in relation to various phases of plant nutrition, Thomas *et al.* (37) determined the fate of radioactive sulfur supplied by fumigation as sulfur dioxide and in the nutrient solution as sodium sulfate. High initial accumulation of sulfur occurred in the leaves followed by a steady lowering as it was redistributed. During ripening, 60 to 80 per cent of the sulfur was translocated to the grain. Conversion of most of the sulfur dioxide and sulfate to organic form occurred rapidly. For purposes of translocation, organic sulfur in the leaves was changed to sulfate and then changed back to the organic form in roots and grain.

*Oxygen*.—Vlamis & Davis (38) compared the salt uptake and oxygen consumption of excised and intact roots of rice, barley, and tomato at oxygen tensions ranging from 0.2 to 100 per cent. They also determined the growth behavior of these three plants in water-logged versus aerated soil, and in culture solutions supplied with air, nitrogen, carbon dioxide, and methane gas. While the excised roots of rice showed very little bromide accumulation at an oxygen tension of 0.2 per cent, the intact roots (tops attached) showed but little decrease of bromide intake when supplied with nitrogen over that occurring when aerated with air. In water-logged soils rice grew well, and in culture solutions supplied with nitrogen and methane, growth was good. These results imply that while, like other plants, rice roots require oxygen for salt accumulation, they apparently derive this needed oxygen from the tops and thus thrive under conditions of oxygen starvation in the root medium. Tomato plants, on the other hand, failed to grow under low oxygen conditions in the root medium, and barley was intermediate in its behavior. Methane actually stimulated rice root growth over that with air. Carbon dioxide was toxic to all three plants, indicating an effect in addition to oxygen exclusion.

The effects of oxygen concentrations, ranging from 0 to 16 p.p.m. in nutrient solutions, on the absorption of calcium, phosphorus, and potassium by tomatoes and by soybeans were studied by Pepkowitz & Shive (39). Soybeans were shown to have a higher oxygen requirement than tomatoes under the conditions of these tests. Maximum absorption of calcium, phosphorus, and potassium was at 16 p.p.m. oxygen for the soybeans, but at 8 p.p.m. for the tomato. At 16 p.p.m. oxygen, total absorption and absorption rate of the tomato were less



than at 8 p.p.m. It was also found that absorption of potassium was less depressed at low oxygen tensions than that of calcium and phosphorus. Climatic conditions favoring high transpiration rate accelerated the absorption of these ions. In further work on the effects of oxygen level, Pepkowitz, Gilbert & Shive (40) determined the organic acid content of the tops and roots of oat plants grown with 0 to 16 p.p.m. oxygen, and also with and without nitrate. More organic acids were found in the plants grown at lower oxygen levels. This is apparently due to incomplete breakdown of these when oxygen is lacking. The plants supplied with nitrate also showed higher organic acid content.

In order to determine the effect of oxygen concentrations on new root development, Boynton & Compton (41) grew young apple, peach, and prune trees for short periods in nutrient solutions aerated with gas mixtures containing from 5 to 20 per cent oxygen. A decrease in oxygen pressure to three quarters that of air caused marked decreases in new root development of these three types of trees.

*Nutrient interrelations.*—Interest in the effects of one ion on the absorption of another continues. In work with excised barley roots, Viets (42) found that a ratio of thirty calcium ions to one of potassium was necessary before a depressing effect on potassium absorption occurred. In lower concentrations, calcium as well as magnesium, strontium, barium, and aluminum increased potassium and bromide accumulation. The effects of calcium were found to operate at various temperatures and also with roots grown previously in high calcium solutions. Under anaerobiosis, no effect of the polyvalent cations was observed but of course absorption is decreased under these conditions. It is suggested that calcium in some manner alters the permeability of the plasma membrane or promotes some necessary reaction in this membrane resulting in increased absorption. Beeson, Lyon & Barrentine (43) grew tomato plants in a sand culture series supplied with forty-three different nutrient solutions. These solutions differed in the relative proportions of calcium, magnesium, and potassium that they contained. The total equivalent concentration, however, was the same in all. The nitrate, phosphate, and sulfate concentrations were the same in all solutions as were the traces of added boron, manganese, zinc, copper, and iron. Both leaves and fruit were analyzed for inorganic constituents. Increases of calcium, potassium, and magnesium in the nutrient solution were all reflected in increases of these, respectively, in the leaves. Phosphorus increased with magnesium

but nitrogen and sulfur contents were not materially influenced by cationic variations. Calcium increased in the leaf when potassium decreased. Increases in calcium slightly decreased the potassium in the leaves but did not affect the potassium in the fruit.

Rose & McCalla (44) report experiments with wheat grown in nutrient solutions in which the effects of limiting nitrogen, calcium, and potassium on plant growth and total mineral uptake were determined. They state that

Although limiting nitrogen and calcium had marked effects on the ratios of the various ions absorbed, anion: cation ratio was not affected. Limiting potassium, however, caused an increase in anion: cation ratio. . . . Limiting calcium had the least effect on uptake of nutrients. . . . Limiting potassium had no effect on percentage of anions but decreased the total weight absorbed.

Bower & Pierre (45) made a study on a high lime soil of Iowa to determine why, in the face of apparently ample amounts of exchangeable potassium, some crops respond to potassium fertilization and others do not. They found that the soil solution extracted from this soil after incubation for one month showed a calcium plus magnesium to potassium ratio of 483. Sweet clover and buckwheat, which do not respond to potassium fertilization on this soil, use large amounts of calcium and magnesium in relation to potassium and hence would tend to lower the divalent ion concentration in the soil solution, thus permitting more ready absorption of potassium. On the other hand, corn, which responds on this soil to potassium fertilization, uses small amounts of calcium and magnesium and thus the higher ratio of calcium plus magnesium to potassium in the soil solution resulting from this situation makes it difficult for this plant to secure ample potassium.

While evidence continues to accumulate concerning nutrient interrelations, there are many conflicting data, and no over-all principles have emerged which harmonize these divergent results.

### SALT TOLERANCE OF PLANTS

Various studies of salt effects on plants continue to appear from the United States Department of Agriculture, Regional Salinity Laboratory at Riverside, California. In a general discussion of saline soils, Magistad & Christiansen (46) summarize some of the more salient features of crop growth in relation to salt. In relation to concentration, the evidence indicates that in many instances reduced growth is mainly due to osmotic effects, that is, inability of the plant

to absorb sufficient water for maximum growth. However, certain plants show special sensitivity to certain ions and the statement is made that "At concentrations less than 2 or 3 atmospheres the nature of the salt and the ratio of one ion to another in the soil or culture solution may affect plant growth more than does the total concentration in atmospheres." They state at another point that "in concentrations exceeding 3 or more atmospheres, the effect of any ion on plant growth is mainly proportional to the extent to which this ion has contributed to the total osmotic concentration."

The effects of climate on salt tolerance of milo, cotton, alfalfa, barley, tomatoes, onions, navy beans, garden beets, and carrots were studied by Magistad *et al.* (47) by setting up replicate experiments at three locations in California differing markedly in mean temperature and humidity. To base culture solutions, having an osmotic concentration of 0.4 atmospheres, were added enough chlorides or sulfates of sodium, calcium, and magnesium to bring the osmotic values to 2.4 and 4.4 atmospheres, respectively. Other solutions contained mixtures of chlorides and sulfates in amounts sufficient to give an osmotic concentration of 4.4. While with many plants, yield was depressed more in the hot climate at a given salt concentration than in the cooler climate, this was not true of other plants. Alfalfa, for example, did not show nearly as great a temperature coefficient as onions. Growth depression caused by increased concentrations of salt tended to be linear in character and no great differences were observable between chloride and sulfate at equal osmotic concentrations.

In an effort to separate the various physiological effects of salts on plants, Long (48) grew approach-grafted tomato plants (single top, two root systems) in variously paired solutions and compared the behavior of roots and tops with those of ungrafted and grafted tomatoes grown in other nutrient solutions. He concluded that salt injury to plants is probably of two kinds: (a) that resulting from increased water tension in the plant; (b) direct deleterious effects of salt on protoplasm and membranes.

The effects of various soil moisture tensions in soils to which 1,000, 2,000, and 4,000 p.p.m. of sodium chloride had been added were studied by Ayers, Wadleigh & Magistad (49). Red kidney beans were used as the test plant. Using soil weight as well as tensiometers as a guide the soils in 10-gallon pots were differentially watered. In one series the plants were allowed to show severe moisture stress before being watered. In another series, the plants were watered

frequently to keep the moisture tension consistently low. In a third series, the plants were watered so as to create an intermediate condition. The beans were adversely affected by all salt additions. Yields in the untreated control as well as in the salt-treated soils were less where water tension was increased. The lowest yield was in the soil with highest salt and where the plants were stressed the most for water. While it is difficult to evaluate and separate the various factors operative in an experiment of this sort, one practical fact stands out, namely, that a given amount of salt in the soil will prove more detrimental if the soil is allowed to approach the wilting point prior to an irrigation than when irrigated in such a way as to prevent periodic moisture stresses. To determine the effects of variable moisture tensions and of salts on the plant, Wadleigh, Gauch & Davies (50) studied the fluctuation of starch in the leaves of red kidney bean plants grown under conditions similar to those described by Ayers, Wadleigh & Magistad (49). The starch determinations were made at successive growth intervals. In general there was less starch in the leaves of the "dry" plants than in those grown on soils kept wet. Following an irrigation, the starch in the "dry" plants built up rapidly, then fell as moisture again became limiting. The presence of 0.2 per cent sodium chloride in the soil decreased the percentage of starch in both the dry and wet series as compared, respectively, with that in the zero salt cultures. These effects on starch are interpreted as resulting from the increased moisture tension in the plant. This may be induced either by drying the soil to near the wilting point before irrigating or by adding salt which increases the difficulty of water uptake.

In order to correlate the results obtained under sand and solution culture conditions with those of soils, Magistad & Reitemeier (51) calculated from soil solution data the theoretical salt concentration at the wilting point of a series of seventeen soils taken from locations where crop growth ranged from very good to failure. They state:

the relationship between plant growth and osmotic pressure of soil solution was similar to and of the same order as that obtained in sand culture and solution culture experiments. Above 40 atmospheres concentration the soils were barren. Normally fertile irrigated soils had a soil solution concentration at wilting percentages of 1.3 to 1.8 atmospheres, conductance values ( $K \times 10^5$ ) of 200 to 350, 2,000 to 4,000 ppm and 30 to 50 m.c. per liter of salts.

In experiments to determine the comparative toxicity of the chlorides of sodium, magnesium, and calcium, and of the sulfates of

sodium and magnesium, Gauch & Wadleigh (52) grew red kidney bean plants to the flowering stage in nutrient solutions of these salts having osmotic pressures from 1.5 to 4.5 atm. The salts were added to a basal nutrient solution of 0.5 atm. osmotic pressure. While there was decreased plant growth with all salts—the yield decreases being essentially proportional to the salt concentrations—the decrease in growth was sharper with the magnesium salts than with the sodium and calcium salts. This indicates a specific toxicity of magnesium apart from osmotic effects. Sodium and calcium salts in isoosmotic concentration were alike in their effects.

The effects of sodium chloride, sodium sulfate, and calcium chloride on the growth of Punjab flax in sand cultures were determined by Hayward & Spurr (53). All salts on an osmotic basis were about equal in their growth-inhibiting effects. Flax was found to be moderately salt tolerant, reasonably good yields being obtained at an osmotic pressure of 2.5 atmospheres. The quality of seed in terms of size, oil content, iodine number, and crude protein was affected to only a slight degree by salt concentration, but yield of seed was reduced by all concentrations of salt. Anatomical studies of the flax stems indicated that increased salt concentrations decreased the growth rate of the cells of the secondary xylem, and decreased the number and size of the phloem fibers.

Wadleigh & Gauch (54) reported salt tolerance studies with guayule plants employing nutrient solutions containing sodium sulfate, sodium chloride, calcium chloride, and magnesium chloride at 1, 2, and 3 atm. osmotic pressure. This plant was found especially sensitive to magnesium, the plants being killed at an osmotic concentration of 1.5 atmospheres. The plants were more sensitive to sodium than to calcium salts and at high concentrations more sensitive to sulfate than chloride.

As regards plant symptoms of salt injury, Magistad & Christiansen (46) state that, save in high concentrations, a dwarfing effect is the usual first evidence of salt injury.

On the basis of controlled work as well as field observations, the comparative salt tolerances of many plants are now known in a general way. Among the more salt sensitive plants are peaches, beans, field peas, oats, and wheat. Among the more tolerant plants are barley, tomatoes, cotton, alfalfa, milo, sugar beets, garden beets, a number of grasses, and date palms.

## NUTRITION IN RELATION TO FRUITFULNESS AND FRUIT QUALITY

The relation of plant nutrition to fruitfulness and fruit quality is a very practical problem and continues to receive increasing attention. Several interesting papers have appeared during the year bearing on some one or more aspects of the relation of nutrition and climatic variables to yield and quality.

Wadleigh (55) carried out a very interesting piece of work with cotton grown in sand cultures in the greenhouse supplied with four nitrate levels varying from 8 to 225 p.p.m. of nitrogen. Growth and flowering behavior were charted throughout and plants from each of the four nitrate levels were harvested when blossoms first began to appear, again forty days later, and at the termination of the experiment. The various vegetative plant parts were weighed, dried, and studied with regard to nitrogen and carbohydrate fractions. The bolls and seed were studied with regard to yield of lint and seed, number of seed per boll, chemical make-up of seed, and fiber length. In the low-nitrogen series, boll formation exerted such a drain on the organic reserves of the plant that vegetative growth ceased for a considerable period. This was not nearly as marked in the higher nitrogen series. In addition to cessation of vegetative growth following boll setting in low-nitrogen plants, abscission of young bolls and squares took place as soon as the number of bolls already set had introduced nitrogen requirements which approached the limits of the nitrogen supplying capacity of the plant. For this reason, it is highly desirable that in early stages of growth cotton be supplied with abundant nitrogen. The results in general are in harmony with and lend support to current concepts of the relation of nitrogen to carbohydrate and the various manifestations associated therewith. It was found that carbohydrates were in general lower in the higher nitrogen plants, and save in the low-nitrogen series the reserve carbohydrate was on the low side. Boll production in the low-nitrogen series was limited by lack of nitrogen reserves, and all subsequently formed bolls, young squares, and terminal buds of fruiting branches abscised. The writer states, "The abscission process . . . appears to act as a 'safety valve,' in that all bolls are shed which could not be adequately developed at the obtaining level of metabolism of the plant." Probably some growth regulating substance actuates this process. Due to high nitrogen, lower light intensity in the greenhouse, and considerable hot weather which would increase respiration rate, carbohydrate reserves were con-

sidered inadequate for maximum boll setting. The lint percentage of the bolls tended to decrease with an increase in nitrogen supply to plants. The protein content of the seeds increased markedly with treatments favoring increased nitrogen reserves in the plant. The oil content of the seed decreased with decrease in carbohydrate. On the basis of existing knowledge of plant biochemistry and the results of this investigation, Wadleigh has presented a diagram showing the major biochemical processes and equilibria in the cotton plant in relation to quality and quantity of plant product.

Eaton & Joham (56), in sand culture experiments with cotton, determined the effects of defruiting on subsequent growth, mineral uptake, and the sugar and starch contents of roots and leaves. The defruited plants, in the twenty-two days which elapsed after this operation, made more top growth, more fibrous root growth, and accumulated more bromine per unit green fresh weight in the fibrous roots and in the leaves, and also accumulated more nitrogen and potassium in the fibrous roots than did the controls. Total sugar and starch were higher in the fibrous and tap roots of the defruited plants than in the controls but in the leaves no difference was evident. The latter effect is attributed to the new top growth made by the defruited plants as compared with the controls. Many others have noted a slowing or stoppage of vegetative growth and nutrient intake following fruiting not only in cotton but in other plants. The present investigation indicates that this is due to failure of sufficient carbohydrate to reach the roots with heavier fruiting which in turn slows mineral uptake. Lack of carbohydrate and mineral uptake in turn slows or stops vegetative development.

In most nutritional work, control of all the major factors affecting plant growth is seldom realized and thus a rounded picture is not obtained. An interesting piece of work by Went (57) with tomatoes grown in air-conditioned greenhouses appeared this year. This is the first of what will probably be a series of papers and concerns principally the effect of varying temperatures and humidity on growth rate and fruiting. The tomato plants were grown in gravel cultures (two-gallon containers) supplied with nutrient solution, and stem elongation was in the main used as the measure of growth. In preliminary work, it was found that at a constant temperature of 26.5° C. most of the stem elongation took place at night, although if night temperatures were reduced to 10° C., then some growth took place in the early morning when temperatures were increased to 17° C. Relative

humidity between 75 per cent and 45 per cent had no effect on growth rate so long as moisture supply was not limiting. Changes from high to low humidity produced transitory changes in growth rate. No significant effect of day length was found at constant temperatures. Under artificial light of only 450 f.c., the growth rate for short periods was comparable to that of daylight.

At various maintained temperatures, growth was found to be nil at 5° C. and to rise steadily from 17° to 26.5° C. When the night temperature was dropped below that of the day, faster growth resulted than at a constant temperature. Thus, with a day temperature of 26.5° C. (eight hours), and a night temperature of 20° C. (sixteen hours), growth was more rapid than at a constant temperature of 26.5° C. This same behavior was found to hold for fruiting. Stage of growth of the plant alters these various optima. These effects indicate some sort of thermoperiodicity and are considered as due to the predominance of two different processes, of which the dark process has a lower temperature optimum than the light process. It is postulated that thermoperiodicity is a general phenomenon in higher plants.

Arnon & Hoagland (58) studied the effects of various nutrient conditions on the inorganic composition of tomato fruit and found that, except under extreme conditions of nutrient deficiency, the fruits were quite constant in composition. Where potassium or phosphorus was deficient, however, marked effects on both composition and size of fruit resulted. In studies of the effects of defloration it was found that with tomatoes somewhat deficient in potash and phosphorus, leaf content of these elements was not decreased as much where no fruit was allowed to develop. In other words, developing fruit makes considerable demands on vegetative parts for phosphorus and potassium and deficiency symptoms will show in leaves sooner in fruiting plants than in nonfruiting plants. Defloration also produced peculiar growth effects indicative of upset metabolism probably involving hormones. Accumulations of starch, sugar, and organic nitrogen were found in the stems and petioles.

The effects of nitrogen, phosphorus, and potassium variations on citrus fruit quality have been reported by Chapman, Brown & Liebig (59). These results were obtained with navel and valencia orange trees growing out of doors in sand and solution cultures of maintained but various concentrations of these three elements. The indications are that fruit quality is more affected by phosphorus and potassium than by nitrogen. Wide variations in nitrogen produced little effect



on size, texture, and juice characteristics whereas wide variation in potash or phosphorus produced quite marked effects. High potash made for large, coarse fruit with thick skins, low juice percentage, and with a higher acid and lower vitamin C content. The effects of phosphorus were just the reverse, high levels of this element produced smooth, solid, high juice fruit of low acid content. Summarizing eight years' field experiments with Arizona grapefruit, Finch (60) concludes that high nitrogen levels during the summer and fall months make for rough, coarse grapefruit of high acidity and low vitamin C. Low nitrogen levels, however, during this same period, make for fruit of improved quality. In the light of the results obtained by the writer and his co-workers, using controlled conditions of nutrition, it would appear that the changed ratio of nitrogen to phosphorus and possibly to other constituents in the foliage and fruit, caused by nitrogen starvation, is a more likely explanation of Finch's results with grapefruit than a specific nutritional effect of nitrogen.

The effect of nutrition on the vitamin content of various edible plant products continues to receive attention. Lyon, Beeson & Ellis (61) grew tomatoes in nutrient media deficient in various minor elements. Although fruit production was decreased by lack of manganese, zinc, copper, and molybdenum, their deficiency had no significant effect on ascorbic acid, riboflavin, or provitamin A. Fruit from iron deficient cultures contained 30 per cent more ascorbic acid. In further work, Lyon & Parks (62) found no lack of ascorbic acid in boron deficient tomato fruit. On the other hand, Harmer & Sherman (63) found, in a Michigan soil, that correction of manganese deficiency increased the ascorbic acid in spinach, oats, and Sudan grass.

In work on skin color of potatoes, Sparks (64) found that iron, iron and copper, and iron, copper, and manganese, in field trials, produced darker red skin color than where these elements were not added. It was also found, in further work (65), that skin thickness was increased by copper, iron, and manganese treatment of the soil.

In a study of the effects of nutritional variables on the rubber content of guayule, Bonner (66) grew this plant out of doors in gravel cultures for eight and a half months. He found that lack of nitrogen reduced rubber accumulation more than any other treatment. Phosphate deficient plants also accumulated less rubber. On the other hand, variations in calcium, potassium, and sulfate were without effect.

In co-operative fertilizer experiments conducted in Virginia, Oklahoma, and Georgia, the effects of variations in nitrogen, phosphorus,

potassium, and calcium on the ascorbic acid content of turnip greens were studied by Reder, Ascham & Eheart (67). Potassium fertilizer decreased ascorbic acid in each experiment. The results were not consistent with the other fertilizers and it was concluded that climatic variations, particularly light intensity and rainfall, exert considerable influence on ascorbic acid formation.

Wilcox & Woodbridge (68) have reported that slight excesses of boron in apple fruit causes, in storage, a browning of the flesh about the core and also around the stem end. This may occur without any evidence of injury to the leaves and twigs of the tree. Boron contents of 25 p.p.m. or higher (dry weight basis) in the fruit are associated with this condition. Since a content of 6 to 7 p.p.m. boron is associated with deficiency, it is evident that the range between deficiency and excess is not great.

#### TISSUE TESTING FOR THE DIAGNOSIS OF NUTRIENT STATUS

The diagnosis of nutritional status by tissue analysis rests on the assumption that for any given plant part or functional unit of given age and species, there is both a minimal and maximal amount of each nutrient or some certain ratio between several or all the nutrients which is required for best performance. If this postulate is sound, then the development of reliable methods is a matter of carrying out sufficient experimental work to establish proper standards, and to determine the significance of departures from these standards in terms of yield and quality of plant product.

Enough evidence is on hand to indicate that, while the absolute total of a given element in a given functional unit is not without value from a diagnostic point of view, in terms of universal application the amount of a given element or compound in relation to certain other elements and organic end products or intermediates may be the best criterion. In this connection, one calls to mind the work of Somers *et al.* (29, 30), in which the question of deficiency and excess of iron and manganese is more a matter of the balance between these elements than the absolute totals. It may be that, as regards calcium and potassium, or calcium and boron, or nitrogen and phosphate, where in each case there is evidence of interaction, it is both the balance and the absolute totals which count. As knowledge of plant nutrition, of soil chemistry, of soil-plant interrelations, and correlative data between plant tests and fertilizer responses grow, it is reasonable to expect that methods of increased reliability will emerge.

In studies with citrus, Chapman, Brown & Rayner (69), found that, while symptoms of deficiency and excess, observed visually, are in most cases sufficiently specific to enable positive diagnosis, the early, incipient symptoms are so general and nonspecific that only by means of chemical tests is it possible to diagnose early stages. Exception to this occurs in the case of zinc, manganese, and magnesium deficiencies. With each of these, a few leaves on the tree will show characteristic symptoms in the early stages and resort to chemical tests is unnecessary. In order to determine the reliability of tissue tests as a measure of phosphorus and potassium deficiency and excess, bearing orange trees are being grown out of doors in water cultures containing graded amounts of these elements. Results to date indicate that the leaf is the most practical plant part to use for the test. Except during the blossoming period it reflects the major differences in the culture solutions. The relationship, however, between the amounts of the element in the leaf and in the nutrient solution is not linear. Leaves of the same age show some variation from year to year due, probably, to variations of weather conditions and of crop volumes.

In studies of leaf analysis with apples, Boynton, Cain & Compton (70) found that with potash, leaves of comparable age from the same orchards where management and fertilization practices were essentially constant showed variations in 1942 over that in 1941. In the case of nitrogen, Boynton & Burrell (71) found that in two different locations the total nitrogen of apple leaves, as correlated with yields, indicated a higher leaf requirement for this element in one location than in another.

Atkinson, Patry & Wright (72) used the Thornton tissue testing method on tomatoes and potatoes growing on differentially fertilized plots and found a fair degree of correlation between the yield increases from various fertilizers and the plant tissue tests. Kalin (73) determined the amounts of total phosphorus in the leaves and stems of tomato plants growing in solutions of graded phosphate levels. Increased phosphate in the nutrient solutions was reflected in total phosphorus increases in both stems and leaves, though the increases were not directly proportional to those of the culture media. Studies to determine the effects of age of leaf, fruiting, and fertilizer treatment on the nitrogen, phosphorus, potassium, ash, calcium, magnesium, manganese, and boron content of Tung tree leaves were reported on by Drosdoff (74). Results in line with those generally found in other plants were obtained.

## RELATION OF NUTRITION TO DISEASE RESISTANCE

Another practical phase of plant nutrition is the relation of the nutritional status to susceptibility to virus, bacteria, and fungal infection. Enough evidence is at hand to indicate the importance of this field and a partial review of this subject has been given by Wingard (75). Shear & Wingard (76) suggest that the increased severity of wilt infection in corn seedlings deficient in potassium is owing to increased nitrate content of the tracheal sap when potassium is deficient. The causal organism, *Phytophthora stewartii*, lives almost entirely in the tracheal tubes during the early stages of invasion and is apparently dependent upon inorganic nitrogen for its parasitic existence. When potassium is not deficient, there is less nitrate in the tracheal sap and thus conditions are less favorable for this organism.

Studying the effects of nitrogen and phosphorus fertilization on root rot of cotton, Blank (77) found that high rates of nitrogen fertilization significantly decreased infection in two out of three years. Phosphorus was without effect on one soil but increased the disease at another location in two out of three years.

Klotz & Sokoloff (78, 79, 80) got indications that exposure of roots of avocado and citrus seedlings to nitrite increased their susceptibility to attack by *Phytophthora* species.

The writer has repeatedly noted increased susceptibility of citrus species to disease under conditions of nutrient unbalance, having found (81) that too high potassium in relation to calcium made orange trees on sour root more susceptible to brown rot gummosis, and the fruit more susceptible to "water rot" and navel end rot than where potassium was lower and calcium higher. High phosphate in the root medium favored root infection by *Thielavia basicola*. When the pH of these cultures was lowered from 5.0 to 4.0, the growth of this organism was discouraged even in the presence of high phosphate and normal root growth became possible.

There are many ways in which nutrition may affect disease due to organisms, as has been brought out by Wingard (75). The answer to many perplexing production problems may lie in further research in this general field.

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# THE CHEMISTRY AND METABOLISM OF BACTERIA

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It has been necessary to confine the material to be discussed in this review to certain aspects only of the very broad subject. This course has been dictated not only by the limitations of the reviewer but also necessarily by the exigencies of the moment, and by the consideration that certain portions of the subject will undoubtedly be reviewed under other headings. Thus, many of the contributions to bacterial nutrition and to the use of bacteria for purposes of bioassay can appropriately be included in the more general subjects of vitamins, amino acids, etc. The present review is limited to (a) a discussion of certain aspects of the chemical composition of the bacterial cell and some of its products, (b) that portion of the current work on bacterial nutrition which relates to new and unidentified growth requirements, and (c) some phases of chemotherapy.

## COMPLEXITY OF THE BACTERIAL CELL

One of the most remarkable properties shared by many varieties of bacteria is the existence of a multiplicity of immunological types for each species of organism. In a number of instances, for example, the pneumococci in which there are more than thirty such different types, the differences have been found to be concerned with the existence of chemically different polysaccharides which make up the mucoid capsules surrounding these organisms. Moreover, under certain conditions, these bacteria may undergo a degenerative change or "dissociation," in which the capsules and the immunological type specificity disappear. With the loss of the capsule, virulence also disappears. Colonies of such degenerated pneumococci are smaller than those formed by the encapsulated cells and have lost the glossy surface characteristic of the latter. The terms "smooth" (S) and "rough" (R) are commonly used to describe the original form and its variant. In general, rough forms from all types of pneumococci are indistinguishable either culturally or immunologically.

By certain procedures it is possible to reverse this change and to obtain smooth forms from the degenerate rough forms. Under normal



conditions the original type specificity is regained, that is, a rough Type II reverts to a smooth Type II. However, Griffith (1) in 1928 showed that a rough culture obtained from Type II could sometimes be converted to a smooth form with different specificity (e.g., Type III) by injecting mice with a small inoculum of living rough Type II and a large quantity of heat-killed smooth Type III pneumococci. The Type III smooth, virulent organisms so obtained could be maintained indefinitely on suitable media, and it thus appeared that a pneumococcus of one type could be permanently changed to another type by passing through an intermediate rough form. These experiments, adequately confirmed, were extended by Dawson & Sia (2) who devised an *in vitro* method of accomplishing the same result also using living R forms and heat-killed S forms. Still further refinements were introduced by Alloway (3) in which Berkefeld filtered, cell-free extracts of the S cells were substituted for the whole, killed organisms.

Avery, MacLeod & McCarty (4) have now announced the isolation from smooth Type III pneumococci of a substance, provisionally identified as a polymerized desoxyribonucleic acid, which in minute amount possesses the property of inducing the R  $\rightarrow$  S transformation involving change in type specificity. As little as 0.003  $\mu$ g. in 2.25 cc. of culture fluid was effective. Ultracentrifugation and cataphoresis gave results supporting the conclusion that the nucleic acid polymer was the active substance. Ultraviolet absorption spectra were characteristic of nucleic acid. A tentative estimate of the molecular weight, based on the physical data obtained, led to the figure 500,000. Solutions of the substance were relatively devoid of precipitability by immune sera capable of reacting in high dilution with pneumococcus protein or with Type III specific carbohydrate. In other words, it appears that a polymer of a nucleic acid may be incorporated into a living, degraded cell, and will endow the cell with a property never previously possessed, namely, the ability to produce a capsule composed of a complex polysaccharide entirely different in structure from that produced by the smooth organism from which the degraded form was originally derived. When thus induced the function is permanent, and the nucleic acid itself is also reproduced in cell division. The importance of these observations can scarcely be overestimated and stimulates speculation concerning such matters as the chemical basis for specificity in nucleic acids, and the genetic implications presented by the ability to induce permanent mutation in a cell by the introduction of a chemical substance. Such speculation may well include considerations of the

relation of this phenomenon to the sequence of events following the introduction of a filterable virus (or a bacteriophage particle) into a susceptible cell. A brief consideration of the facts now accumulated concerning the chemical composition of the viruses may bring this relation into perspective.

It is generally recognized that the most outstanding characteristic of filterable viruses as a group is their failure to multiply outside of the host cell. Beyond this single common property there are wide differences in size and in chemical complexity. From the upper extreme represented by the visible elementary bodies of vaccinia and psittacosis viruses and (for the sake of uniformity of concept) the bacillary forms called rickettsiae, down to the crystalline plant viruses and such agents as poliomyelitis and foot and mouth disease viruses which approach the dimensions of large protein molecules, there are representatives of a wide range of sizes. Similarly, in regard to chemical complexity, while all viruses contain nucleoprotein and the smallest may consist of single molecules of this material alone, the larger ones approach the bacterial cell in complexity, containing lipid, polysaccharides, and even certain enzymes. Thus as a group viruses may reasonably be considered as living cells deficient in a range of vital functions which must be supplied by a suitable host cell. For example, the larger viruses may be devoid of oxidative mechanisms only, but retain many synthetic capabilities. The latter drop out progressively as size and complexity decrease until nothing remains in the smaller viruses but a large molecule of nucleoprotein multiplying as a result of the combined vital processes of the host cell.

Considerable definite information relating to the chemical composition of the largest and the smallest of the viruses has been available for some time. Information has now been provided by Taylor and others about viruses of intermediate size (5, 6, 7), namely influenza viruses A and B and swine influenza virus. Lipoid components varied from 21 to 24 per cent among the three, and included neutral fat, phospholipid, and cholesterol. The remainder consisted of polysaccharide, protein, and desoxyribonucleic acid. Even these viruses of intermediate size, therefore, are still relatively complex entities. It would be interesting to know whether they still possess certain enzymes such as phosphatase which has been found to be present in vaccinia virus (8) though absent from small viruses such as the polyhedral virus of *Lymantria monacha* L. (9), and tobacco mosaic and bushy stunt viruses (10). Williams, Schlenk & Eppright (11) re-

ported the absence of the various components of the B vitamin complex from tobacco mosaic, tobacco necrosis, and bushy stunt viruses and from a strain of influenza A virus. They believe such absence carries implications of the inanimate nature of viruses. In the light of the above discussion it seems probable that the cellular functions dependent upon these readily extractible substances may well be missing from even the largest viruses, which may yet retain so complex a make up and properties so varied as to bring their inanimate nature into considerable question.

The one component common to all viruses, therefore, is nucleoprotein, while in the case of the substance inducing variation in the pneumococcus, nucleic acid alone is involved. In the latter case a synthetic and purposeful change is brought about, i.e., the ability to produce a polysaccharide capsule rendering the cell resistant to certain destructive influences. On the other hand, so far as is known, viruses lead to degenerative changes in the host cell and eventually to its death. Conceivably, therefore, the crucial difference between the transplanted "gene" and the smallest virus may lie in the specificity of the protein which is present in the latter, or to which the former probably attaches itself in the cell. In the one case the protein heterologous to the cell, renders the particle a completely foreign parasitic molecule, which eventually becomes injurious as it accumulates. In the other instance, the cell's own protein combines with the new nucleic acid, and the resulting homologous nucleoprotein takes its place in the normal economy of the cell and confers a new property upon it. In any case, these considerations again indicate the enormous complexity of cellular and bacterial protoplasm, and emphasize our complete ignorance of the many and varied metabolic processes which proceed inside the cell wall.

As further illustrations of the great complexity of the bacterial cell a few instances from the contributions of the current year may be mentioned, selected primarily because of some bearing on medical bacteriology.

Stockinger, Ackerman & Carpenter, for example, reported extensive studies of the composition of the gonococcus (12) and its products (13). They described two nucleoprotein fractions obtained by extraction of mass cultures. One of these contained considerable combined lipid. Carbohydrate was found in three different forms, but a polysaccharide having type specific properties was not detected. A variety of lipoidal constituents were present, among which a lecithin,

a cephalin, and sphingomyelin were identified. From broth in which the gonococcus had been grown, they isolated a protein, believed to be a degradation product of the cellular nucleoprotein. It was moderately toxic to animals and possessed immunological specificity as measured by complement fixation. Boor & Miller (14) prepared carbohydrate-lipid complexes from a number of strains of gonococcus and meningococcus. These "glucolipids" were also moderately toxic for animals and were antigenic in rabbits. Sera so produced precipitated both the glucolipid itself and the carbohydrate component separated by acid hydrolysis. These complexes, therefore, have properties similar to compounds of the same sort prepared earlier by Boivin & Mesrobianu (15, 16) from a variety of gram negative bacilli.

Kabat, Kaiser & Sikorski (17) have prepared a polysaccharide from Type I meningococci which is electrophoretically homogeneous, and which is type-specific. It is weakly but definitely antigenic in man. Direct comparison with an earlier product obtained by Scherp & Rake (18) indicates that the avoidance of acid or alkali during the preparation of the more recent material has resulted in a somewhat purer and more nearly natural antigenic substance.

An antigenic capsular polysaccharide has been obtained from *Cl. perfringens* by Svec & McCoy (31). It appears to be common to most members of the *perfringens* group, regardless of their toxigenic properties.

The tremendous amount of investigation on the chemistry of tuberculin carried out in the last two decades has been reviewed during the year by Seibert (19), particularly in regard to carbohydrates, nucleic acid, and protein. Corper & Cohn (20) have compared the tuberculoprotein obtained from autolyzed tubercle bacilli with that present in culture filtrates, and found similar properties, together with greater purity, in the former. Chargaff & Moore (32) described the isolation of glycogen from tubercle bacilli. The molecular weight of the material was shown to be of the order of 12 to 13 million. Anderson and his collaborators (21, 22, 23) have added to an already extensive investigation of the lipids of the tubercle bacillus, and carried out similar studies (24, 25) with *Phytomonas tumefaciens*, incited by the possibility that some of its lipid material may be responsible for the ability of the organism to induce plant galls. An unidentified fat acid with the formula  $C_{20}H_{40}O_2$  was isolated. It is believed to possess a branched chain.

The capsular material of certain hemolytic streptococci, identified

some time ago as hyaluronic acid, has aroused considerable interest as to its possible relation to the virulence of this organism. Some evidence pointing in this direction has been adduced by Kass & Seastone (26). Other bacteria and even some strains of hemolytic streptococci may secrete an enzyme, hyaluronidase, capable of hydrolyzing this acid. This enzyme appears capable of acting on the tissues of the body at the site of infection in such a way as to lead to a rapid dissemination of particulate material (India ink, bacteria) and is an important member of a group of substances called "spreading factors" which share this property. This subject was reviewed in 1942 by Duran-Reynals (27). In the current year, however, Crowley (28) has examined a considerable number of strains of hemolytic streptococci for hyaluronidase production, and finds no relation between this substance and virulence. Similar results have been obtained by Humphrey (29) for various pneumococci, and by Evans (30) for the Welch bacillus. The significance, therefore, of the production of hyaluronic acid by some bacteria, and of hyaluronidase by others is not yet clear.

#### GROWTH REQUIREMENTS OF BACTERIA

As already stated, it is not proposed to review completely the voluminous literature which continues to accumulate on bacterial growth requirements. The significant developments of the year will quite surely be reviewed elsewhere in this volume. It may not be out of place to point out at this time the accuracy of the prediction made by the writer more than twenty years ago (33) that definite knowledge of bacterial growth requirements would quite certainly supply information of considerable value in fields other than bacteriology. The incredibly close relation between the nutrition of animals and that of microorganisms, and the present concept of certain types of chemotherapeutic action more than justify the earlier optimism.

The bacterial culture still appears to offer the most convenient tool for the definition of unrecognized nutritional substances, for the existence of which there is ample evidence. The factor which seems nearest to complete elucidation is "folic acid." The rather chaotic facts bearing on the chemistry and physiology of this and related substances have been recently reviewed (34). Probably nothing can bring greater testimony to the change in status of bacterial nutrition in the last twenty-five years than the present state of affairs in which most of the pharmaceutical companies in the country are competing

for first place in clearing up this tangle. The writer recalls somewhat grimly the difficulties encountered in 1920 while attempting to enlist cooperation in getting a hundred pounds of casein hydrolyzed with sulfuric acid, from which the first of the unrecognized growth factors for bacteria (methionine) was eventually isolated.

There are a number of observations of other probably new growth factors. Their relationship to each other, to "folic acid," and to factors already more accurately defined must await chemical identification. Happold and his collaborators (35, 36) have described a substance occurring in acid-hydrolyzed casein, and more abundantly in liver extract, which is required for the growth of certain strains of *C. diphtheriae* and of *L. casei*. From liver extract a considerable degree of purification has been achieved, and certain of the properties of the material have been defined. It is not replaced by folic acid. A second factor greatly stimulating early growth of *L. casei* appears also in liver extract, and behaves in such a manner as to lead the authors to believe it may be identical with a growth factor for *L. casei* described by Pollack & Lindner (37) as present in Wilson's bacteriological peptone. The latter authors describe the substance as being stable at 100° between pH 2 and 11, but reduced in potency to half its original strength by heating at pH 12 and destroyed by 0.7 N sulfuric acid. Happold's factor, however, is stated to be adsorbed by norite at pH 3, whereas the substance in peptone is said not to be adsorbed to any extent by charcoal (Darco G-60) nor by a variety of other adsorbents between pH 3.0 and 8.0. A third factor for *L. casei* is described by Happold as occurring in liver extract in a combined and inactive form. Gentle hydrolysis splits it off in an active condition.

Smith (38) reported the presence in yeast extract of a growth stimulant for *Streptococcus lactis*. It failed to precipitate with the heavy metal salts and was not adsorbed by charcoal (Darco) or fullers' earth.

Sprince & Woolley (39) point out the similarity between the second Happold factor (above) for *L. casei*, the Pollack & Lindner factor for the same organism, the Smith factor for *S. lactis*, and a factor for the hemolytic streptococcus previously described by Woolley (40). Some evidence for this similarity is presented, and the name "streptogenin" is tentatively suggested for the substance. The efficacy of a number of concentrates of "solubilized liver extract" prepared in various ways was compared on the three varieties of bacteria and in each case the relative recovery of active material was the same for all

three organisms. One of the preparations employed was not made from liver extract but from partially acid-hydrolyzed vitamin-free casein. The "per cents of recovered activity" for the three organisms are stated to be 17, 19, and 18 respectively. It is not clear to the reviewer what value, in this case, is taken as 100 per cent, since both the original casein and its complete acid hydrolysate are said to be inert. In any event decision on the relationship of these several growth manifestations must await the isolation or better characterization of an active material.

Evidence for the existence in tomato juice of an unidentified stimulant for growth of *L. arabinosus* has been given by Kuiken and collaborators (41). It is prepared by adsorption on charcoal and elution with a mixture of pyridine, alcohol, and water. It is stable to strong acid hydrolysis, but its properties are not further described.

Ballentine *et al.* (42) have studied a growth factor for *Cl. perfringens* occurring in yeast extract. After precipitation by lead hydroxide, the substance was recovered with hydrogen sulfide and impurities removed with the resin Amberlite IR-4 followed by norite adsorption. It was stated to be precipitable by mercury and by picric acid but not by a variety of other reagents. It was stable to cold normal acid and alkali though inactivated by both in five minutes of boiling.

Welton, Stokinger & Carpenter (43) have presented the composition of a synthetic medium for the growth of the gonococcus. One of the components is indole-3-acetic acid, and thus by implication this substance is essential for the growth of the organism and must be considered to be a potential bacterial growth factor. So far as the reviewer is aware, this is the first available evidence that a plant auxin may function as a growth stimulant for bacteria, and the observation deserves further amplification.

Gould (44) has found that certain passage strains of the gonococcus develop a requirement for glutathione. This appears to be the first instance of the identification of this substance as a bacterial growth factor. The strains which depend upon the presence of this material for growth are inhibited by low concentrations of cystine, possibly through a blocking or competitive action in some essential system.

Finally, the presence of two chloroform-soluble components of liver extract which promote the growth of *Lactobacillus helveticus* and *Streptococcus lactis* has been described by Barton-Wright, Emery & Robinson (45). These substances are not destroyed by nitrous acid, or by acetylation or benzylation. They are unique among the

factors mentioned in this review because of their solubility in chloroform. The authors promise a further description. It is possible that fatty acids or other lipid material may be at least partially responsible for the effects noted. Oleic acid and oleates are known to act as growth stimulants or depressors (according to concentration) for the diphtheria bacillus (46) and the tetanus bacillus (47). Moreover, Kodicek & Worden (48) have recently directed attention to a variety of changes in the growth of *L. helveticus* obtainable with materials which are chloroform-soluble and almost certainly occur in liver preparations. Their results showed that growth of the organism is augmented by palmitic and stearic acids, though inhibited by oleic, linoleic, and linolenic acids. The effect of linoleic acid is reversed by either cholesterol or lecithin.

#### CHEMOTHERAPEUTIC ACTION

Evidence continues to accumulate in support of the view, first expressed by Fildes (49), that chemotherapeutic substances operate by blocking some specific chemical grouping necessary for the successful growth of the microorganism. The empirical observation of the value of sulfanilamide and other related drugs as therapeutic agents was followed by the discovery of a specific inhibitory substance in tissue extracts, and later by the provisional identification of this substance as *p*-aminobenzoic acid (50). Subsequently this compound was shown to be essential for the growth of certain bacteria, and therefore presumably to function as a component of some metabolic system. Sulfanilamide appears to be sufficiently similar in structure to combine at the same point, but the next step in the reaction fails to occur and the metabolism of the cell is arrested. The relatively low toxicity of the sulfonamides for animals may be due either to production in the cells of sufficient *p*-aminobenzoic acid to prevent the blocking or to the ability of tissues to replace the blocked function by some alternative mechanism.

A wide variety of compounds of the general nature of *p*-aminobenzoic acid and sulfanilamide, but with various substituent groups at different positions, have been tested both as bacteriostatic substances and as inhibitors of sulfonamide stasis (51 to 55), and certain general relationships appear to have been established. The nature of the process which normally involves *p*-aminobenzoic acid remains undetermined. There is evidence, however, that two of the sulfonamides,



sulfapyridine, and sulfathiazole, also block a second function which is concerned with a nicotinamide-stimulated respiratory process (56, 57, 58). This latter effect is not prevented by *p*-aminobenzoic acid. Moreover, in recent work, Reed, Orr & Reed (59) describe differences between *in vitro* and *in vivo* effectiveness of the sulfonamides on the gas gangrene group of bacteria and intimate that evidence is to be forthcoming that two types of drug inhibition may exist.

Differences between species of bacteria in relation to sulfa drug susceptibility are of considerable interest. That within the same species may be found both susceptible and resistant strains, and that the former can apparently acquire the properties of the latter either *in vitro* or *in vivo*, are facts of the utmost practical importance. Resistance to the action of one of the sulfonamides usually extends to all the others, and once established, the property seems to be permanent. In the test tube, the change may be brought about by exposure to sublethal concentrations of the drug. The same mechanism probably operates in the body. It is consequently not surprising that evidence has already begun to accumulate that certain human infections originally yielding in most instances to drug therapy may become increasingly refractory to this type of treatment. The widespread use of small doses of sulfadiazine for the prophylactic checking of such bacteria as the meningococcus, gonococcus, and streptococcus would appear especially suited to the elimination of susceptible strains and their replacement by others, equally virulent, but so altered metabolically as to be highly resistant to the sulfonamides. That this is more than a theoretical possibility is illustrated by the fact (60) that in 1940-41 about 70 per cent of cases of gonorrhea treated in one Boston clinic were promptly cured by sulfonamide therapy, while the remaining 30 per cent were unaffected. At the present time the proportions are approximately reversed, and but for the timely introduction of penicillin it seems probable that the chemotherapy of this infection would shortly have become relatively ineffective.

Experimental demonstration of the acquisition of drug resistance *in vitro* has been made by numerous workers (e.g., 61, 62, 63). The possibly complex nature of the change is indicated by the observation (64) that whereas pneumococci which have acquired a high degree of resistance to sulfonamides appear to remain permanently in that state, partial resistance may be lost rather promptly when contact with sulfonamide is discontinued. In some instances, at any rate, resistance appears to be due to increased production of *p*-aminobenzoic acid

(65), but not all naturally resistant organisms produce extracellular inhibitors (66). Fortunately resistance to the sulfonamides does not parallel that against penicillin (67). Information on the mode of action of this antibiotic is not yet available.

A peptide containing ten or twelve glutamic acid residues linked to the carboxyl group of *p*-aminobenzoic acid has recently been isolated from yeast (68). It is not antagonistic to the sulfonamides until hydrolyzed by acid.

In view of the clinical success attending the use of the sulfonamide drugs, and of the development of a well supported theory of their mode of action, it is not surprising that a number of attempts have been made to explore the possibility of using derivatives of other known growth factors as chemotherapeutic agents. Thus, McIlwain (69) has shown that pyridine sulfonic acid inhibits the growth of organisms requiring nicotinic acid or nicotinamide and recognized three "types" of inhibition depending upon the individual circumstances. The sulfonic analogue of pantothenic acid, "pantoyltaurine," has been prepared by Snell (70, 71) and shown to inhibit the growth of bacteria which require pantothenic acid. The inhibition was reversed by additions of the latter. Similar observations were reported independently by Kuhn, Wieland & Moller (72) who also observed the specific antagonistic effect of pantothenic acid and, in addition, noted a partial annulment by  $\beta$ -alanine. McIlwain (73) has obtained similar results and shown that the effect is inhibited by pantothenic acid and by  $\beta$ -alanine. Certain strains of streptococci were resistant to the action of pantoyltaurine. This property was entirely unrelated to sulfonamide resistance (74). Neither was it related to the requirement of pantothenic acid for growth, which was common to all strains examined. Resistant strains could be rendered susceptible by salicylate and it is suggested that this fact may be evidence for the existence of alternative mechanisms for the same function, one involving pantothenic acid, the second blocked in some way by salicylate. In the case of the diphtheria bacillus, McIlwain was able to effect the transposition of strains susceptible to the action of pantoyltaurine into resistant forms by "training" those originally requiring pantothenic acid to produce their own (75). This was accomplished by gradual withdrawal of pantothenic acid and demonstrates that drug resistant strains may develop without the presence of the drug itself. Certain of the above observations have been extended by quantitative studies of the fermentation of glucose by streptococci under circumstances in which

pantothenic acid and pantoyltaurine can be shown to exert antagonistic effects (76, 77).

McIlwain & Hawking (78) have shown that in rats, pantoyltaurine acts as a moderately effective chemotherapeutic agent against hemolytic streptococci. It is ineffective in mice, which have a higher level of pantothenic acid in the blood. The possibility is pointed out that pantoyltaurine may be of use in human streptococcus infection, since the concentration of pantothenic acid in human blood is even lower than in rat blood.

Nielsen & Johansen (79, 80) have shown that with a yeast, stimulation of growth induced by  $\beta$ -alanine is prevented by  $\beta$ -amino butyric acid and by isoserine, but not by taurine.

The pyridine analogue of thiamine, "pyrithiamine," shown by Woolley & White (81) to induce symptoms of thiamine deficiency when fed to mice, was later found by the same authors (82) to inhibit the growth of bacteria requiring thiamine or its component moieties. Again, the effect was reversible by the natural vitamin. A resistant strain of yeast was obtained by growth in the presence of the inhibitor (83). This strain, however, still required thiamine or its pyrimidine portion as a growth factor.

Studies on possible modifications of biotin are beginning to appear. Desthiobiotin, described by du Vigneaud and his associates (84) can replace biotin for growth of a yeast but not of *L. casei*. It was later shown (85) that in the case of the latter organism, the substance in suitable concentration inhibits growth, acting as a specific antibiotin. Biotin sulfone and imidazolidone caproic acid exert a similar effect (86) while a number of other derivatives and related compounds either partially replaced biotin or were inert. The ability of certain of these compounds to replace biotin from its union with avidin is also reported. Lilly & Leonian (87) investigated the effect of desthiobiotin on a large number of microorganisms, which they were able to classify as to their behavior toward the compound. Antibiotin activity was manifested toward some but not all varieties unable to use desthiobiotin.

Modification of riboflavin by substitution of two methyl groups by chlorine yielded a product showing bacterial growth inhibitory properties for certain organisms (88). The inhibition was reversed by riboflavin.

That modifications even of the amino acids may result in compounds which interfere with normal bacterial growth is evident from

McIlwain's (89) experiments with sulfonic analogues of glycine, alanine, valine, leucine, and aspartic acid. Delay or inhibition of bacterial growth resulted from their presence, but the effect was readily reversed by natural  $\alpha$ -amino acids, regardless of specific structure. Fox, Fling & Bollenbach (90) obtained considerable inhibition of growth of *L. arabinosus*, which requires *l*-leucine, by means of the unnatural isomer. This observation should be amplified, since synthetic amino acids have been universally employed in bacterial growth experiments. In the work cited the proportion of *d* to *l* form is given as 200 to 1. Since general experience has led to the belief that the forms not occurring in nature are sometimes used quite as readily as the natural forms, in other instances are inert and occasionally have exhibited anomalous behavior (see for example 91-93) a further quantitative as well as qualitative investigation of the phenomenon is desirable.

The facts thus far available in connection with the effect of chemotherapeutic agents on diseases due to filterable viruses are generally in harmony both with the commonly held explanation of sulfonamide action and with the view that viruses are dependent upon host cells for many of their functions. Thus, while it has been known for some time that the sulfonamides were moderately effective against such large viruses as psittacosis and lymphogranuloma, they are completely inert in the case of the smaller agents (94 to 97). The typhus fever rickettsia appears to be moderately susceptible to penicillin, and, curiously, to *p*-aminobenzoic acid (98), a fact for which no ready explanation seems obvious. It is possible that as more knowledge of the vital processes remaining to intracellular parasites becomes available, some form of systematic approach to their chemotherapy may be found. Thus far, the empirical attack has been entirely negative although a wide variety of organic compounds of various types has been investigated (99).

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# THE CHEMISTRY OF ANTIBIOTIC SUBSTANCES OTHER THAN PENICILLIN

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*Introductory remarks.*—By reason of a regulation designed to ensure the better prosecution of the war effort, the first article in the *Annual Review of Biochemistry* to deal exclusively with a comparatively new but rapidly growing field in microbiological chemistry cannot include anything about the most remarkable substance in that field, namely penicillin. This is the less to be regretted since penicillin ought undoubtedly to have a review to itself when publication upon its purification and chemical nature is again permitted. The few chemical facts concerning it which had been published before the imposition of the ban have been summarised (1). The present review is admittedly written from the standpoint of the organic chemist in the hope that it may provide a basis for future generalisations concerning the relationship between molecular structure and antibiotic action. The subject was last reviewed from a wider angle by Dubos in 1942 (2) and briefly mentioned by van Niel (3) a year later.

*Definition of antibiotic substance.*—For the present purpose an antibiotic substance is defined, subject to the qualifications set out below, as a soluble, organic substance, which is produced by a microorganism from a harmless constituent (or constituents) of a medium and which has been found to be markedly inhibitory to the growth or activity of a second microorganism, when it is dissolved in a medium otherwise suitable for the normal growth or activity of this second microorganism. The necessary qualifications are:

- (a) The substance has been isolated and tested in a pure state.
- (b) The term microorganism includes only yeasts, molds, actinomycetes, and bacteria. Microscopic algae are not here considered, although a recent preliminary report (4) indicates that a future reviewer may well have to take them into account. Preliminary surveys of the antibacterial substances of the higher green plants have also been made (5, 6) but it is by no means certain that any very potent substance will emerge from such studies, since bacterial diseases of plants are mostly due to vigorously growing members of the genera *Pseudomonas* and *Bacterium* (7), which are Gram-negative bacteria, notoriously insusceptible to chemical inhibition. It has never been



convincingly shown that plants have any real chemical defence against such bacteria, save perhaps for certain glycosides in bark (8).

(c) The smallest concentration of the antibiotic substance in the medium producing a measurable effect upon the inhibited organism *in vitro* shall be of the order of 50 p.p.m. or less. A less active substance is unlikely to be of any therapeutic value, nor would it impart any detectable antibacterial properties to the metabolic solution containing it unless formed in very considerable yield by the inhibiting organism.

*Historical.*—A brief historical sketch seems fitting, if only for the sake of completeness, although much of the ground has been covered before in more comprehensive reviews. Antagonism between different microbial species has long attracted attention and has given rise to a voluminous literature, reviewed a few years ago by Porter & Carter (9) and again, more extensively, by Waksman (10). It has of course long been surmised that when microorganisms compete in a natural substrate, such as vegetation, soil, water, sewage, milk, it may happen that one species derives a considerable advantage from its ability to make a soluble substance which is toxic to other microorganisms present. But our definition covers rather more than this, for it occasionally happens that a microorganism, apparently not so equipped to gain an advantage over its natural competitors, nevertheless may produce a substance which is extremely toxic to a microorganism it is unlikely to have to compete with in nature, since the latter is known to need quite different conditions for normal growth or even survival. It should be remembered that in nature molds do not usually compete with bacteria (11) but with each other. Consequently many of the recent intensive efforts to find new antibiotics, especially those concerned with the production of an antibacterial agent by a mold, must if successful be in the nature of happy accidents, should the substance isolated have a very selective bacteriostatic action, but no fungistatic or general toxic action. The most famous of all these happy accidents, and indeed the only real one which has occurred so far, was of course the discovery of penicillin by Fleming in 1929 (12), for there is no good reason to suppose that *Penicillium notatum* Westling, the responsible mold, ever has to compete with pathogenic cocci, or even saprophytic bacteria resembling them, in its native habitats.<sup>1</sup>

<sup>1</sup> According to Thom (13) Westling's original culture was "found on rotting branches of *Hysopus* in Norway."

The literature concerning antibiotic substances up to the discovery of penicillin in 1929 has been adequately reviewed by Buchanan & Fulmer (14). Reading this review one is struck by the absence of any effect which had been traced to a definite chemical substance, and to the frequency with which *Pseudomonas* species appear as inhibiting organisms. The striking antibacterial effect of *Pseudomonas pyocyanea* was not properly analysed into its components until 1941 (15), but the inhibitory action of its blue, water- and chloroform-soluble pigment, pyocyanine, obtained in crystalline form as long ago as 1860, had been recognised in 1932 (16). Pyocyanine therefore was the first antibiotic substance to be discovered.<sup>2</sup> As far as the writer is aware, the striking effect ascribed to the very common, water-borne organism *Pseudomonas fluorescens* (19), which does not produce pyocyanine, has never been satisfactorily accounted for,<sup>3</sup> although the inhibitory substance is chemically stable and is formed when the organism is grown in very simple synthetic media (19).

Omitting all reference to intervening studies on penicillin before 1940, and passing over a remarkable instance of antagonism in milk discovered in 1933 (20), which unaccountably seems to have escaped further notice until quite recently (*vide infra*), we come to the isolation of tyrothricin by Dubos in 1939 (21, 22). This spectacular result was the culmination of researches, extending over several years, which aimed at the deliberate exploitation of microbial antagonisms in soil. As early as 1931 Dubos & Avery (23) had isolated a bacillus from peat soil which would decompose the specific capsular polysaccharide of *Pneumococcus* Type III when this carbohydrate was supplied as the sole source of carbon to a mineral salt medium. A slight shift of viewpoint later led Dubos to the conclusion that "the addition to soil of living cultures of Gram-positive cocci would result in the development of a selective flora capable of attacking the living cells of these bacterial species." Experimenting along these lines he isolated a motile, Gram-positive, aerobic, spore-bearing bacillus, later identified as *B. brevis* (24), which was capable of lysing suspensions of living staphylococci. More important still, the lysis was primarily

<sup>2</sup> At the same time a definite effect upon the anthrax bacillus was claimed for the pure bacterial pigment prodigiosine ( $C_{20}H_{25}ON_3$ , formed by *Chromobacterium prodigiosum*), but since this pigment is not soluble in water, and had to be tested under rather artificial conditions, it has not been included in this survey. Its structural formula is known (17).

<sup>3</sup> By no means all strains of *Ps. fluorescens* show this effect (18).

due, not to an enzyme, but to the action of a toxic chemical agent found in solution in autolysates of the soil bacillus when the latter had been grown in an amino acid medium. Later the active agent, tyrothricin, was separated from an inactive protein by taking advantage of its solubility in organic solvents (22) and further resolved into two distinct crystalline substances (25), namely gramicidin and tyrocidine hydrochloride, both polypeptides, which had very remarkable, but qualitatively different, inhibitory effects upon susceptible bacteria. Gramicidin is in fact about the most active antibiotic agent known against pneumococci *in vitro* (26). Dubos' 1939 papers and the re-evaluation of the chemotherapeutic possibilities of penicillin published by Florey *et al.* in the following year (27) provoked widespread research in this hitherto dormant section of microbiology, with the result that there are now at least fourteen crystalline antibiotic substances within the scope of our definition, of which seven are of known structural formula (in some instances elucidated before 1939), and two of partly known structure. There is also at least one amorphous substance which has been obtained in a reasonably pure state, together with several which, at the moment (October 1944) exist only as crude preparations of sufficient antibacterial activity to be worthy of mention.

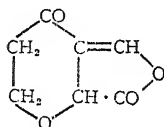
#### LIST OF ANTIBIOTIC SUBSTANCES

Compounds are arranged below according to the method of classification used in Richter's *Lexikon der Kohlenstoff-Verbindungen*, the description of each individual being set out in the following order: formula and molecular weight (in brackets); chemical name and structure; trivial name or names;<sup>4</sup> physical properties; microorganisms producing it, together with cultural conditions, method of isolation and yield; chief arguments in favour of the structure assigned; biological activity exhibited.

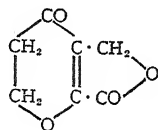
##### *Section 1; compounds containing carbon, hydrogen, and oxygen*

<sup>4</sup> If the structure is reasonably well authenticated these names are listed in alphabetical order. Otherwise the name first given to the pure compound is adopted and other names follow between brackets. Is it too much to hope that future workers in this field will refrain from giving a natural product a definitive name ending in "-in" until it has been obtained in a reasonably pure state; and further be willing to forego the use of such name if it becomes clear that another has purified and named the same compound first? The time-honoured, if unwritten, conventions of organic chemistry ought surely to be observed here as in other branches of Biochemistry.

only;  $C_7$  group.— $C_7H_6O_4$  (154); anhydro-3-hydroxymethylene-tetrahydro- $\gamma$ -pyrone-2-carboxylic acid (structure Ia) (28); clavacin (29), clavatin (30), claviformin (31), patulin (28);<sup>5</sup> colourless, m.p.  $111^\circ$ , optically inactive, neutral, and readily soluble; *Penicillium claviforme* (31, 32), *P. patulum* Bainier (28), *P. expansum* (33), an unnamed *Penicillium* (34), *Aspergillus clavatus* (29, 30, 35, 35a), *Gymnoascus* species (34); from a synthetic medium containing glucose at  $24^\circ$ ; by evaporation of the metabolism solution *in vacuo* and ether extraction; 0.3 gm. per l. (*P. claviforme*), 1 gm. per l. (*P. patulum*).



Ia

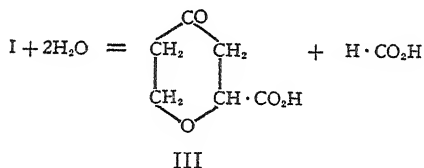


Ib



II

The chief arguments in favour of structure Ia are as follows (28): Acid hydrolysis yields one molecule of formic acid (and no other volatile acid) and, in small yield, another acid, identified with synthetic inactive tetrahydro- $\gamma$ -pyrone-2-carboxylic acid, III, the synthesis of which has not yet been published. Thus:



Granting that the metabolic product really is a derivative of  $\gamma$ -pyrone, and the evidence on this point would have been stronger if it had been shown that the small yield of III could be ascribed to its instability under the conditions of the hydrolysis, the point of attachment of the remaining carbon atom must be at the 3-position since reduction by hydrogen in presence of platinum oxide, followed by hydriodic acid, yielded  $\beta$ -methylcaproic acid and its  $\gamma$ -hydroxy-derivative. In order to account for the neutral properties of the substance the carboxyl

<sup>5</sup> Probably never before has a crystalline natural product of small molecular weight received as many as four different names within two years of its discovery. Faced with this array the reviewer is constrained to take the line of least resistance and proceed as if the compound had never been named at all (see footnote 4).

has to be lactonised in the manner shown, and indeed the substance does behave as a typical unsaturated lactone. Numerous other reduction experiments yielded results perfectly consistent with structure Ia. Confirmatory evidence has also been provided (36) by the conversion of the metabolic product by straightforward transformations into  $\beta$ -n-propylbutyrolactone and  $\gamma$ -keto- $\beta$ -methyl-n-hexoic acid, each of which contains all the original seven carbon atoms. The position of the double bond in the lactone ring is still uncertain (cf. 36) since the optical inactivity of the compound, and an application to it of the arguments recently used to establish that the cardiac aglucones are  $\Delta^{\alpha\beta}$ - rather than  $\Delta^{\beta\gamma}$ -unsaturated lactones, particularly the formation of lactones rather than desoxyacids on catalytic hydrogenation (cf. 37), would favour Ib rather than Ia. Furthermore, an easily established equilibrium between Ia and Ib in neutral aqueous solution cannot lightly be assumed because the evidence obtained in the comprehensive studies of the behaviour of unsaturated lactones referred to above is against such a view (cf. 37). A further point of some dubiety is the explanation given for the easy acylation and etherification of the metabolic product, presumably by preliminary enolisation of the carbonyl group of the 6-membered ring although proof of this is lacking. It should not be overlooked that  $\Delta^{\beta\gamma}$ -angelicalactone (II with  $R = H_2$ , structurally similar to Ia) contains a very reactive  $\alpha$ -methylene group (41a).

The compound inhibits both Gram-positive and Gram-negative bacteria without selective action, is toxic to animal tissues (31), and has marked fungistatic properties (33, 38). Its hydrolysis product III has no comparable antibacterial action, nor have two other  $C_7$ -compounds produced by *P. patulum* (39), viz. gentisic acid (hydroquinone-carboxylic acid) and the corresponding alcohol (2:5: $\omega$ -trihydroxytoluene). The substance provides yet another instance of an unsaturated lactone with a marked action on biological systems. Others include  $\Delta^{\beta\gamma}$ -angelicalactone (II,  $R = H_2$ ) and certain of its derivatives (II with  $R = :CH \cdot C_6H_4 \cdot OH$  etc.) which are anthelmintics exhibiting also a depressant action on muscular and nervous tissue (40, 41), and the well known cardiac glycosides of the digitalis-strophanthus group.

*Section 1; compounds containing carbon, hydrogen, and oxygen only; C<sub>8</sub> group.*—(a).  $C_8H_6O_8$  (198); structure not yet fully elucidated; puberulic acid (42); colourless, m.p. 316–318°; optically inactive, dibasic and water-soluble; *Penicillium puberulum* Bainier and

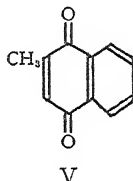
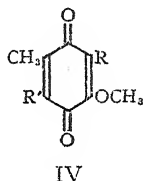
*P. aurantio-virens* Biourge (42), *P. cyclopium-iridicatum* series, *P. Johannioli* Zaleski (43); from a synthetic medium containing glucose at 24°, by precipitation as a nickel complex and acetylation of the crude acid liberated from this complex; 0.1 gm. per l. (from *P. aurantio-virens*). In spite of extensive analytical studies (42, 44) all that can be said with certainty is that puberulic acid is not a dihydroxybenzenedicarboxylic acid, although it resembles one in many respects. It contains at least one true carboxyl group and two hydroxy groups, but it is not possible to write any convincing structure for it containing the benzene ring. It has a moderate inhibitory action against Gram-positive bacteria only (43).

(b).  $C_8H_8O_4$  (168); 3-hydroxy-4-methoxy-2:5-toluquinone (structure IV with  $R = OH$ ,  $R' = H$ ); fumigatin; maroon, m.p. 116°, water-soluble (45); *Aspergillus fumigatus* Fresenius [one strain (45)], *A. fumigatus* particularly one strain No. 84 (46); from a synthetic medium containing glucose at 24–28°; by aeration, acidification and extraction with chloroform; 0.15 gm. per l. (45), 0.04 gm. per l. (46); synthesis (47, 48). Fumigatin is moderately active against Gram-positive bacteria (much more so than toluquinone itself) but not against Gram-negative bacteria (49). Other derivatives of toluquinone, and even of benzoquinone, containing methoxyl groups, have been found to be much more active than fumigatin against *Staphylococcus aureus* (50), particularly when  $R = R' = H$ , and when  $R = H$  and  $R' = OCH_3$  in structure IV. The methyl group attached to the nucleus may also be replaced by other alkyl groups without sensible alteration of antibacterial activity (51), and the methoxyl group does not really seem to be essential for that activity, since 2-methylnaphthoquinone (structure V) is highly active<sup>6</sup> (52, 53). All highly active true *p*-quinones which however have no therapeutic possibilities (53 a) were found to possess an oxidation-reduction potential, at pH 7, numerically less than  $-0.10$  or  $+0.15$  volt, but not all quinones the oxidation-reduction potentials of which fell between these limits showed marked antibacterial activity (53), possibly because they were chemically unstable in complex bacteriological media at 37° (54).

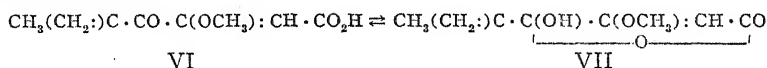
(c).  $C_8H_{10}O_4$  (170);  $\gamma$ -keto- $\beta$ -methoxy- $\delta$ -methylene- $\Delta^a$ -hexenoic acid (tautomeric structures VI and VII); penicillic acid; colourless, m.p. 87° (anhydrous), 64–65° ( $+1H_2O$ ); optically inactive, mono-

<sup>6</sup> Certain bacteria are known to produce the fat-soluble vitamin- $K_2$  but the simple water-soluble form of this vitamin (structure V) has not yet been detected as a metabolic product of a microorganism.

basic acid, readily soluble in water (55); *Penicillium puberulum* Bainier, noteworthy because it was isolated from mouldy maize (56, 57), *P. cyclopium* Westling, one strain only (55); from a synthetic medium containing glucose at 24°, by evaporation *in vacuo* and recrystallisation; 2 gm. per l. (from *P. cyclopium*). Penicillic acid



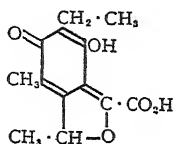
contains one methoxyl group which is lost, together with carbon dioxide, by reaction with phenylhydrazine to yield the phenylpyrazoline phenylhydrazone derived from an unsaturated- $\alpha$  diketone conclusively shown to be acetylmethacrylyl. It was also shown to possess a terminal  $\text{:CH}_2$  and on catalytic reduction gave dihydropenicillic acid [VI and VII with  $\text{Me}_2\text{CH}$ . in place of  $\text{Me}(\text{CH}_2\text{:})\text{C}$ .]. The latter yielded methyl dimethylpyruvate on oxidation (a result which both fixed the position of the methoxyl group and indicated the existence of a second double bond), and  $\gamma$ -hydroxy- $\delta$ -methylhexanoic acid on reduction by hydriodic acid. This proves that the carboxyl in penicillic acid is at the end of the chain remote from the terminal double bond. The constitution is in fact extremely well authenticated on the analytical side (55) but a synthesis is very desirable in view of the marked antibacterial activity of what is apparently a simple, open-chain structure.



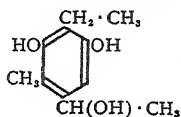
Penicillic acid is active against both Gram-positive and Gram-negative bacteria (58, 59). It appears to be toxic to some extent towards animal tissues (56, 57) but no careful study of its toxicity has yet been published. Dihydropenicillic acid has no marked antibacterial properties.

*Section 1; compounds containing carbon, hydrogen, and oxygen only; C<sub>13</sub> group.*— $\text{C}_{13}\text{H}_{14}\text{O}_5$  (250); anhydro-2-ethyl-5-( $\alpha$ -hydroxy)-ethyl-6-methylresorcylic-4-glyoxylic acid (structure VIII); citrinin; yellow, m.p. 166–170° (decomp.), *l*-rotatory (in alcohol), monobasic acid, almost insoluble in water but readily soluble as the sodium salt

(60); *Penicillium citrinum* Thom (60), *Aspergillus terreus* (61), *Aspergillus* species of the *candidus* group (62), and at least one strain of *P. expansum* (18); from a synthetic medium containing glucose at 28–32°; by acidification and recrystallisation of the precipitate; 0.7 gm. per l. (from *P. citrinum*).



VIII



IX

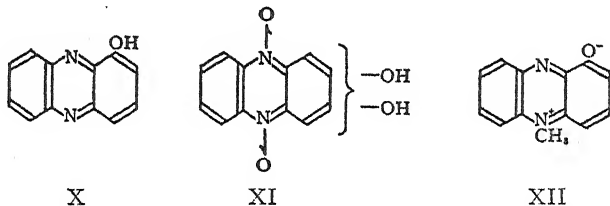
Citrinin has not yet been synthesised. The arguments leading to structure VIII have been summarised (63) and the chief of them are briefly as follows: like the simpler mold metabolic product  $C_7H_6O_4$  described on pages 752–54, citrinin yields one molecule of formic acid on acid hydrolysis, and no other volatile acid. One molecule of carbon dioxide is also evolved and an optically active neutral substance,  $C_{11}H_{16}O_3$ , is formed:  $C_{13}H_{14}O_5 + 2H_2O = H \cdot CO_2H + CO_2 + C_{11}H_{16}O_3$ . This neutral substance yielded by potash fusion a dihydric phenol  $C_9H_{12}O_2$ , which on good and sufficient grounds was thought to be 2-ethyl-4-methylresorcinol (cf. however 64); hence the neutral substance, which also couples with diazotised aromatic amines by virtue of a free position ortho- or para- to a phenolic hydroxyl group, must have structure IX, the side-chain  $\cdot CH_2 \cdot CH_2 \cdot OH$  being debarred because it contains no asymmetric carbon atom. The introduction of a carboxyl group, which is to yield carbon dioxide, and another carbon atom which is to be split off as formic acid on hydrolysis, can convincingly be done in only one way, viz. as  $\cdot C \cdot CO_2H$  attached to the one free position left in the benzene ring of IX; and if it is granted that the colour and stability of citrinin are more consistent with a para- than with an ortho-quinone structure, then VIII is clearly the most probable structure for the pigment. Citrinin is markedly inhibitory towards Gram-positive bacteria only (65, 59).

*Section 1; compounds containing carbon, hydrogen, and oxygen only; C<sub>32</sub> group.*— $C_{32}H_{44}O_8$  (556); structure unknown; helvolic acid (66), fumigacin (46, 67); colourless, m.p. 212°, *l*-rotatory in chloroform, monobasic acid insoluble in water except as sodium salt; *Aspergillus fumigatus* mut. *helvola* Yuill (66), *A. fumigatus* (46,



67); from a synthetic medium at pH 8 containing glucose; by adsorption on charcoal at pH 4 and elution with acetone; 0.4 gm. per 100 l. (66). Beyond the facts that it is a monobasic acid yielding a crystalline methyl ester (68), that it contains three active hydrogen atoms, and is a ketone (67), little is known about the structure of helvolic acid. It is markedly active against Gram-positive bacteria only, and is chiefly bacteriostatic in its action. Its toxicity to animal tissues is not great (66).

*Section 2; compounds containing carbon, hydrogen, oxygen, and nitrogen; C<sub>12</sub> group.*—(a). C<sub>12</sub>H<sub>8</sub>ON<sub>2</sub> (196);  $\alpha$ -hydroxyphenazine (structure X); hemipyocyanine; yellow, m.p. 158°, amphoteric with basic and phenolic properties; *Pseudomonas pyocyanea*, in old cultures (15); synthesis (69, 70) also easily synthesised from pyocyanine. It has a moderately strong antibacterial action, less than that of pyocyanine, but it is also much less toxic and irritating than the latter. Its action is comparable with that of acriflavine (15). According to Stokes *et al.* (71)  $\alpha$ -hydroxyphenazine has considerable fungistatic properties and is more inhibitory towards yeasts and fungi than to bacteria.



(b). C<sub>12</sub>H<sub>8</sub>O<sub>4</sub>N<sub>2</sub> (244); NN'-dioxide of 1:2-dihydroxyphenazine<sup>7</sup> (structure XI); iodinin; deep purple pigment with coppery glint, m.p. 236° (decomp.), insoluble in water and acids, soluble in alkali and phenolic in character; *Chromobacterium iodinum*, on beer-wort agar at 30° for several days; the pigment surrounds the bacteria, is detached by addition of water, collected and crystallised from chloroform; 1 gm. per square metre of solid medium (72). Although by analysis iodinin appears to be a tetrahydroxyphenazine, its colour and non-basic properties exclude this structural possibility. It yielded in fact an unknown dihydroxyphenazine on reduction, which was not identical with the 2:3- or 2:6-derivative. Iodinin could be regenerated from this dihydroxyphenazine by oxidation (74). Since the NN'-dioxide prepared

<sup>7</sup> The position of the hydroxyl groups is perhaps still in doubt (cf. 73).

from  $\alpha$ -hydroxyphenazine showed great similarity to the natural pigment, it was concluded that one hydroxyl group in the latter must occupy the 1-position. The other was assigned to the 2-position chiefly because the absorption spectrum and fluorescent properties of iodinin resembled those of alizarin (1:2-dihydroxyanthraquinone) rather than quinizarin (1:4-dihydroxyanthraquinone). Definite evidence of the co-ordination of at least one hydroxyl with an N-oxidic oxygen atom was obtained. Evidently the exact structure can be settled only by synthesis and this has not yet been achieved.

Iodinin is highly inhibitory towards *Streptococcus haemolyticus*, rather less so against *Staphylococcus aureus*, and is much less active against Gram-negative bacteria (75). McIlwain (74) has prepared a number of other NN'-dioxides which were found to be inhibitory to bacterial growth in concentrations at which their parent diazines were inactive. He also found (73) that the antibacterial action of iodinin against *Streptococcus haemolyticus* may be counteracted by proportionately small concentrations of para-quinones bearing a formal structural analogy to iodinin, e.g., quinizarin, and 2-methylnaphthoquinone (structure V). The inference drawn was that the action of iodinin may be to inhibit systems in the bacterial cell normally concerned with such quinones, which must participate to some degree in the bacterial metabolism. This conclusion ought not to be lightly accepted since McIlwain himself noted that 2-methylnaphthoquinone was inhibitory to streptococcal growth at a concentration of about 0.002 per cent and if, as seems likely, the quinone is more active still against *Staph. aureus* (53), while iodinin is less active against this organism than against *Str. haemolyticus*, the illogical position might arise that the quinone, supposedly a part of some vital mechanism in the cell, would be more toxic towards staphylococci than its antagonist iodinin. Furthermore it has been recognised (75a) that "an inhibition can be antagonised without the antagonist necessarily being a normal component of the reacting system in question."

(c).  $C_{12}H_{20}O_2N_2$  (224); structure unknown; aspergillic acid; colourless, m.p. about  $96^\circ$ , monobasic acid with amphoteric properties, insoluble in water except as the sodium salt; *Aspergillus flavus*, from a tryptone medium, by adsorption on norite charcoal and elution with ether; yield, very small (76). Aspergillic acid is moderately active against Gram-positive and Gram-negative bacteria, most of all against *Staph. aureus* (76, 77). It has a relatively high toxicity for mice, apparently largely confined to the nervous system.

*Section 2; compounds containing carbon, hydrogen, oxygen, and nitrogen; C<sub>13</sub> group.*—C<sub>13</sub>H<sub>10</sub>ON<sub>2</sub> (210); internal phenazonium salt derived from  $\alpha$ -hydroxy-N-methylphenazine (structure XII); pyocyanine; dark blue, red in acid solution, m.p. 133°, water-soluble, and amphoteric; *Pseudomonas pyocyanea*; grown in a special peptone broth at pH 7.4 for six days at 37°; by aeration and chloroform extraction, then re-extracted from the chloroform by acidulated water. This was made alkaline, the pigment re-transferred to chloroform, and finally converted into the picrate (78); yield, 0.01 gm per l. (70); synthesis from  $\alpha$ -hydroxyphenazine (79, 70); finally shown to be an internal salt by Hillemann (80). Pyocyanine has a high general antibacterial activity (15, 71) but is very toxic to animal tissues.

*Section 2; compounds containing carbon, hydrogen, oxygen, and nitrogen; C<sub>41</sub> group.*—C<sub>41</sub>H<sub>56</sub>O<sub>11</sub>N<sub>8</sub> (only approximate) (mol. wt. about 1000, determined cryoscopically); structure unknown; actinomycin A; red, m.p. 250° with slow decomposition, almost insoluble in water, dilute acids, and alkalis, but readily soluble in many organic solvents; highly *l*-rotatory in alcohol (81); *Actinomyces antibioticus*, from a starch-tryptone medium (at pH 7) at 25 to 35°, but the pigment is also formed in lower yield on a synthetic medium; by ether extraction and addition of light petroleum which removes the soluble actinomycin B, a colourless substance not yet purified concerning the antibacterial activity of which some dubiety exists: the residue insoluble in light petroleum is purified by chromatography; 0.1 gm. per l. (81). If the above empirical formula, or one near it, is accepted it would seem that actinomycin contains no methoxyl, but two each of the NCH<sub>3</sub> and C·CH<sub>3</sub> groups. Waksman & Tishler (81) consider actinomycin to be a quinone, largely because of its colour, and its ready and reversible reduction to a pale yellow substance, but this view is not supported by its behaviour on acetylation, which when carried out either reductively or in the ordinary way appears to introduce two acetyl groups. The acetylation of a true para-quinone should introduce one more or two less acetyl groups than the corresponding operation carried out on its quinol, according to whether the quinone undergoes the Thiele-Winter acetylation or not. It is to be hoped that this most interesting compound will be further investigated, for the possibility, evident from its properties and elementary composition, that it contains a polypeptide chain, like gramicidin, as well as a chromophoric group, does not seem to have been considered. Actinomycin is extremely active, bacteriostatically, against certain

Gram-positive bacteria, notably *Sarcina lutea* and *Bacillus subtilis*. It is much less active against the coli-typhoid group of Gram-negative bacteria. It is also highly fungicidal and very toxic to animals (81).

*Section 2; compounds containing carbon, hydrogen, oxygen, and nitrogen; C<sub>146</sub> (approximate) group, and other substances of high molecular weight.*—There are at present only two crystalline compounds to be considered here, viz. gramicidin, and tyrocidine hydrochloride, both formed by *Bacillus brevis* (*vide supra*). Since both are polypeptides that are resistant to the action of proteolytic enzymes, and have been investigated simultaneously, they are conveniently considered together. They may be separated by treatment of tyrothricin with acetone-ether, the soluble part yielding gramicidin when recrystallised from acetone, and the insoluble part, tyrocidine hydrochloride when recrystallised from alcohol containing hydrochloric acid (24). Short descriptions follow: (a). C<sub>146</sub>H<sub>202</sub>O<sub>26</sub>N<sub>30</sub> (approximate) (82); mol. wt. (deduced by stoichiometric reasoning discussed later) 2790 (82), in agreement with values obtained by physical and chemical methods (83); gramicidin; m.p. 230–231°; slightly *d*-rotatory in alcohol, insoluble in water. (b). Empirical formula and molecular weight unknown; contains about 14 per cent nitrogen and 2.7 per cent chlorine (25); tyrocidine hydrochloride; m.p. 240° decomp.; highly *l*-rotatory in alcohol, sparingly soluble in water.

The newest method of protein analysis viz. partition chromatography, carried out on the mixture of acetyl-derivatives of amino acids occurring in a protein hydrolysate after acetylation according to the procedure of Gordon *et al.* (84), has recently been applied to gramicidin and tyrocidine by the discoverers of the method, with on the whole conspicuous success. The definite advantages of this micro-method are that the individual acetyl-derivatives are isolated in a pure state, titrated, and compared with authentic specimens. The chief sources of error, apparently inevitable however carefully the chemical hydrolysis of a protein may be carried out, lie in the destruction, alteration, or partial racemisation of amino acids, particularly tryptophane, before the adsorption stage is reached. As will be seen the errors so caused seemed to be greater with tyrocidine than with gramicidin which contains fewer amino acid residues.

Before the application of partition chromatography to this particular problem, it was known that gramicidin possessed no free carboxyl or amino groups, not even as end-groups, and that it yielded tryptophane, leucine, valine, and alanine on hydrolysis together with an ap-

parently new hydroxy-amino acid (85, 86); but all the nitrogen had not been accounted for. All of the leucine and half of the valine seemed to possess the "unnatural" *d*-configuration. Tyrocidine, on the other hand, contained free  $\alpha$ -amino groups, and both acidic and basic amino acid residues. It yielded also the following on hydrolysis: ammonia, tryptophane, tyrosine, alanine, and phenylalanine, certain of which again possessed the *d*-configuration. This last fact, which may have some bearing on the antibacterial action of the polypeptides (87) is not as remarkable as might appear at first sight, for it is not at all uncommon for micro-organisms to make the "unnatural" optical isomers of well-known structural building units, e.g., acid-fast bacteria produce *d*-arabinose from glycerol (88); ergotinine and other ergot alkaloids, which are definitely products of fungal metabolism, contain *d*-proline (89); the anthrax bacillus and *B. mesentericus vulgatus* produce a polypeptide composed entirely of *d*-(-)-glutamic acid units (90, 91), which has apparently never been tested for its antibacterial activity; and lastly it has recently been reported that *Penicillium resticulosum* produces *dl*-fumarylalanine on a synthetic medium with glucose as sole source of carbon and nitrate as sole source of nitrogen (92). The last named acylpeptide had no appreciable antibacterial action.

The recent analysis of gramicidin (82) was published before that of tyrocidine (93) and the very considerable difficulties encountered with the estimation of tryptophane in the latter perhaps make it probable that the recorded tryptophane content of gramicidin, viz., 40 to 45 per cent, is a little too low. Gordon *et al.* tentatively attribute the discrepancies in the tryptophane content of tyrocidine, destruction of the amino acid being almost complete in some experiments, "to the different course which the interaction of tryptophane and other amino acids may take under the conditions of hydrolysis, when these substances are present as polypeptides rather than as free amino acids or their simple derivatives." Apart from this reservation, the results indicated that gramicidin contained twenty-four (or some multiple of twenty-four) amino acid residues, made up as follows: six each of leucine and tryptophane, five of valine, three of alanine, two of glycine, and two of an unknown hydroxy amino acid (not isolated) perhaps isoserine. In confirmation of previous work, the leucine was found to be largely *d*-, the valine was *dl*-,<sup>8</sup> and the other amino acids had the

<sup>8</sup> If there is an odd number of valine residues in the molecule, some racemisation must occur on hydrolysis for the *dl*-amino acid to be isolated.

usual configuration. The number of nitrogen atoms thus accounted for was thirty, leading to a minimum molecular weight of about 2800. A cyclic polypeptide structure was suggested with twenty-four peptide linkages. The only information at present available concerning the relative situations of the various amino acid residues is that contained in a recent important study by Christensen (94) on the partial acid hydrolysis of gramicidin. An optically inactive form of the dipeptide valylvaline was isolated, composed of approximately equal amounts of *d*-valyl-*d*-valine and *l*-valyl-*l*-valine, together with little, if any, of *d*-valyl-*l*-valine and *l*-valyl-*d*-valine. Thus a valine residue, whenever it occurs adjacent to another valine residue, never has for its neighbour a valine residue of opposite configuration. Since free valine, as well as the stable dipeptide valylvaline, appeared in the early stages of hydrolysis, not every valine residue in the gramicidin molecule occurs adjacent to another valine residue, and the presence of at least five such residues must be inferred (cf. 82).

Tyrocidine has been shown (93) to contain the following amino acid residues (apart from that of tryptophane) the combined nitrogen contents of which in one instance, together with the nitrogen in ammonia evolved after a brief hydrolysis (85), amounted to 81 per cent of the total nitrogen: phenylalanine, leucine, valine, proline, tyrosine, aspartic and glutamic acids, and ornithine. This is the first time that the latter has definitely been recognised as a constituent amino acid of a protein-like substance, and it is believed that ornithine and proline both exist in the intact molecule of tyrocidine, although the presence of citrulline is not excluded. In this instance phenylalanine had the *d*-configuration, but some of the other amino acids isolated were considerably racemised. The minimum number of amino acid residues in tyrocidine cannot yet be deduced with reasonable certainty. Tyrocidine has a marked bactericidal effect on both Gram-positive and Gram-negative bacteria, whereas gramicidin is primarily a bacteriostatic agent, inhibitory to all Gram-positive species except acid-fast bacilli (24).

*Section 3; compounds containing carbon, hydrogen, oxygen, nitrogen, and sulphur; C<sub>13</sub> group.*—C<sub>13</sub>H<sub>14</sub>O<sub>4</sub>N<sub>2</sub>S<sub>2</sub> (326); structure unknown; gliotoxin; colourless, m.p. 221° decomp.; almost insoluble in water and dilute hydrochloric acid, soluble in caustic soda in which it is not stable; highly *l*-rotatory in many organic solvents (95); *Gliocladium fimbriatum* (96, 95), *Aspergillus fumigatus* (46, 67, 97); an unnamed *Penicillium* (98); in a synthetic medium containing sucrose

and ammonium sulphate at pH 3 to 3.5 for four days at 27 to 32°, the culture being shaken; 0.05 gm. per l. [from *G. fimbriatum* (95)]. Nothing has yet been published concerning the molecular structure of gliotoxin save that its ultra-violet absorption spectrum indicates the presence of an indole nucleus (95). When boiled with caustic potash it rapidly loses sulphur as sulphide. A sulphur-free compound,  $C_{13}H_{12}O_2N_2$ , is also formed by reduction with hydriodic acid (99). Gliotoxin is highly active bacteriostatically against Gram-positive, but not against Gram-negative bacteria (26, 100). It is also highly fungicidal, and its first isolation was the result of a research designed to find out why the hyphae of a supposed *Trichoderma* species (later identified as *Gliocladium*) destroyed another fungus when they curled round it, the second fungus being *Rhizoctonia solani* Kühn, a parasite responsible for the damping-off of citrus seedlings (101).

*Other antibiotic substances not yet fully purified.*—The best authenticated are listed below:

(a) *Diplococcin*.—Whitehead (20) noted that milk sometimes contains certain lactic acid-producing streptococci which inhibit the normal action of the very similar bacteria which are used industrially as cheese-starters, and further proved that the inhibition was due to a protein-like substance. The industrial significance of this antagonism has recently been further investigated (102). The inhibiting coccus has also been grown in a semi-synthetic medium containing sucrose, and a proteose-like antibiotic substance has been extracted by chemical means from the cells so obtained (103). Like gramicidin and tyrocidine, the new antibiotic (diplococcin) contained neither sulphur, phosphorus, or polysaccharide. A similar or identical substance seems to be present in the metabolic solution and is responsible for its antibacterial action (103, 104). Diplococcin has a highly selective bacteriostatic action against Gram-positive cocci and *Lactobacillus* species, but is inactive against Gram-negative bacteria. It may have therapeutical possibilities against bovine mastitis, the causative agent of which is *Str. agalactiae* (104).

(b) *Notatin* [also known as penicillin B, penatin, and *E. Coli* factor (105 to 108)].—This protein, produced by *Penicillium notatum* and at one time a source of confusion with respect to the real nature of penicillin, is a glucose oxidase enzyme. It is bactericidal only in the presence of glucose and oxygen, and since the effect is really due to hydrogen peroxide, it does not fall within the definition of an antibiotic substance given at the beginning of this article. Green (109)

has recently shown that xanthine oxidase in presence of xanthine or hypoxanthine and oxygen has a similar action against *Staph. aureus*.

(c) *Inhibitory organic bases formed by Actinomyces species.*—These include streptothricin, streptomycin, and proactinomycin. Streptothricin, a supposedly basic substance, but insoluble in the usual organic solvents, is formed by *Actinomyces lavendulae* on a synthetic starch- or glucose-glycine medium (110, 111). It is active against Gram-negative bacteria of the coli-typhoid group, particularly in an alkaline medium (112). Precise details for its isolation and purification have not yet been released, but it is said not to be unduly toxic to animal tissues and a favourable report concerning its therapeutic possibilities has recently appeared (113). A very similar substance, streptomycin, has been isolated from *A. griseus* culture fluids (114), and appears to be even more consistently active against Gram-negative bacteria than streptothricin (115). A third basic substance, proactinomycin, occurs in the metabolism solution of a *Proactinomyces* species, and can be extracted by ether at pH 10 but not at pH 5. It is markedly active only against Gram-positive bacteria (116).

*Types of structure occurring in antibiotic substances.*—Although of course sweeping generalisations are as yet premature, it is of interest that five well-defined types of structure have been met with so far in those substances, listed above, the chemical properties of which have been investigated in some detail. These structures are: unsaturated  $\gamma$ -lactone with unusual side-chains; true para-quinone; complex pigment with quinone-like structure; phenazine derivative; protein-like substance of small molecular weight and free from sulphur. The first is typically a structure elaborated by molds, the fourth by bacteria (and possibly actinomycetes), and the last, unlike the others, is not that of a metabolic product but of a cellular constituent set free by autolytic or chemical disintegration, e.g., gramicidin, tyrocidine, diplococcin.

*Chemical differences between Gram-positive and Gram-negative bacteria.*—Most antibiotics are highly active against Gram-positive bacteria only, and those substances which are also active against Gram-negative bacteria are usually toxic to animal tissues. Dubos has pointed out that "there is a far greater similarity between tissue cells and Gram-negative organisms, as far as the penetration and toxicity of compounds is concerned than between these cells and Gram-positive cocci. Substances such as penicillin, with low toxicity,



may be expected to have activity confined to Gram-positive organisms, while others like gliotoxin will have wide activity but be of no value for parental therapy because of toxicity for the tissues" (quoted in 117). In spite of this opinion the quest for weakly toxic antibiotics with high activity against Gram-negative bacteria is perhaps not hopeless, for it is a well known fact that the immune serum prepared against a Gram-negative bacillus has a much more marked bactericidal action against that organism, in presence of complement, than in the corresponding case of an anticoccal immune serum prepared against and acting upon a Gram-positive coccus (118). It is perhaps unlikely that any simple substance of small molecular weight will be found to be non-toxic and yet highly active against Gram-negative bacteria, but that a polypeptide-like substance with these properties may exist is conceivably within the bounds of possibility.

"The differential susceptibility of the Gram-positive and Gram-negative species . . . is obviously the result of some fundamental difference in cellular structure" (24). The older theory of the mechanism of the Gram stain (119) was that the isoelectric point of the surface constituents of the Gram-positive cell was between pH 2 to 3 compared with pH about 5 for Gram-negative organisms. Consequently the former had a greater affinity for basic dyes, and this affinity was increased by oxidation with iodine which pushed the isoelectric range still further to the acid side. The chemical nature of these acidic cell constituents remained unknown, but a very recent theory (120) seeks to ascribe the difference between the two types of bacterial cells to the presence of the magnesium salt of ribonucleic acid at the surface of Gram-positive bacteria. It is for example possible to convert a Gram-positive into a Gram-negative organism by extraction with sodium cholate (cf. the lytic action of bile salts on pneumococci) and to restore the Gram-positive character by combination with magnesium ribonucleate. It is to be hoped that this suggestive theory will be extended to provide an explanation of the highly specific mode of action of such antibiotics as penicillin, gramicidin, and certain para-quinones. Another remarkable fact yet to be explained is the striking inhibitory effect of traces of highly unsaturated fatty acids, particularly linoleic and linolenic acids, upon Gram-positive bacteria only (121, 122).

Apart from these straight-chain unsaturated fatty acids, which are of widespread occurrence in lipoids, certain saturated but branched-chain fatty acids obtained from various natural sources seem also to

have a considerable bactericidal action; for although phthioic acid, the toxic hexacosanic acid isolated from the human tubercle bacillus (123) is not ethyldecyldodecylacetic acid as supposed by Stenhagen & Stållberg (124), several saturated fatty acids with branched chains and quaternary carbon atoms have been prepared by Robinson *et al.* (125, 126, 127) in their attempts to synthesise it, and these acids are reported to have marked antibacterial properties. Acids of similar structure occasionally occur in the higher plants also, particularly in the di- and tri-terpene series. It has in fact recently been reported that nordihydroguaiaretic acid (doubtless derived from the guaiaretic acid  $C_{26}H_{24}O_4$  of guaiacum resin) is markedly active against staphylococci and various *Salmonella* species (6). No theory has yet been put forward to account for the surprising bactericidal action of these branched-chain, saturated fatty acids.

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